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A CORRELATION BETWEEN CELL SURVIVAL AND CHROMOSOMAL DAMAGE USING SYNCHRONISED CHINESE HAMSTER OVARY CELLS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A correlation between Cell Survival and Chromosomal damage using synchronised Chinese hamster ovary cells, submitted by Ramesh N. Bhambhani in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Synchronised cell populations are useful in determining whether cellular radiation responses are "age-dependent," in addition to enabling biochemical explorations of the cell. The parameters often investigated when assessing the radiosensitivity of cells *in vitro* are cell survival and chromosomal damage. With the assumption that the two phenomena may be related as a premise, the present investigation attempts to ascertain the role of chromosomal damage in cellular lethality. This is accomplished by measuring the amounts of chromosomal aberrations in differently synchronised cell populations and correlating these data with assays of cell survival on similarly synchronised cell populations.

Investigations with halopyrimidine analogs (5-BUdR, IUdR and others) indicate that they are readily incorporated into the DNA of mammalian cells as well as other organisms. Their incorporation results in a modification of radiosensitivity. This investigation examines the above mentioned end points of radiosensitivity of Chinese hamster ovary cells grown in 5-BUdR for one and two generations, as well.

On the basis of the evidence of a vast array of experiments, the preeminent role of the nucleus as the prime target for radiation-induced damage cannot be doubted. This study lends added evidence to the involvement of the nucleus as one of the targets in radiation-induced damage as well as to the belief that the two parameters of radiosensitivity are "age-dependent." The investigations with the base analogue suggest that the increased radiosensitivity may be related to the incorporation of 5-BUdR in the DNA of the cells.

A strong correlation between chromosomal damage and cellular lethality is demonstrated. However, it is suggested that caution be exercised

in implicating chromosomal damage as the primary cause of radiation-induced cell death until the mechanisms by which these lesions arise and cause cell death are elucidated.

The current investigation utilised two methods of cell synchronisation —mechanical shaking and chemical arrest, which were found not to differ considerably with respect to the two radiation responses studied.

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INTRODUCTION

The irradiation of living cells with ionising radiation incurs molecular perturbations of sufficient magnitude to bring about a general response resulting in the overall disturbance of cellular activity. The law of Bergonie and Tribondeau (1905) states that "radio sensitivity of a tissue is dependent upon the number of undifferentiated cells it contains, the degree of mitotic activity in the tissue and the length of time that the cells remain in active proliferation." Many investigators have subsequently made concerted efforts to learn more about the relationship between the metabolic state of cells and the effectiveness of radiation. The perplexing variables in radiobiological systems, however, have not permitted a precise definition of this relationship.

Recent refinements in mammalian cell culture techniques have, however, permitted a somewhat clearer picture to emerge. The advances in single cell culture, cell-cycle analysis and cell synchronisation have thus enabled certain parameters of radiation damage to become established. In the first place, single cell growth has permitted the delineation of cell survival (ability of single cells to form colonies subsequent to radiation) as a radiation response. Second, cell synchronisation procedures have permitted the selection of cells in specific phases of the cell cycle and have thus proved useful in the study of age-dependent biochemical responses.

Various studies have revealed that the degree of expression of radiation damage, in terms of cell survival and chromosomal aberrations, is "age-dependent", viz., dependent upon the stage of the cell in the cell cycle. In addition, dose-modifying factors suggest that the two responses are correlated. Such information, when combined with a knowledge of the levels

of DNA, RNA and protein syntheses under various experimental conditions, has led to the hypothesis that the nuclear material in living cells is the primary target of ionising radiations (Erikson and Szybalski 1963, Puck and Marcus 1956, Till 1963, and others).

Cell synchronisation procedures should be such that the cells, to be subsequently utilized in radiation response studies, are subjected to the least amount of traumatic stress possible. Two methods, 1) mitotic selection through shaking with subsequent maintenance in mitosis at 4°C, and 2) metaphase arrest with colcemid, have been commonly utilized. One of the objectives of this investigation was to compare these two systems of synchrony in a single strain of Chinese hamster ovary (CHO) cells, with respect both to cell cycle progression and to radiation responses (cell survival and chromosomal damage), subsequent to synchronisation. X-ray induced cellular mortality in mammalian cells has been attributed primarily to chromosomal aberrations caused by X-ray irradiation (Puck 1959, and others). This investigation attempted to ascertain the role of chromosomal damage in cellular lethality by measuring the amounts of chromosomal aberrations in differently synchronised cell populations and correlating these data with measurements of cell survival on similarly synchronised cell populations.

The thymidine (TdR) base analogue 5-bromodeoxyuridine (5-BUdR) is not only incorporated into the nucleic acids of bacteria, plant and mammalian cells (Erikson and Szybalski 1963, Kaplan *et al.* 1962, Kihlman 1963, and others), but also results in an enhancement of X-ray induced damage (Dewey and Humphrey 1963, and others). In chromosomes the increase in damage occurs in specific regions of intense incorporation (Dewey *et al.* 1966). However, the quantitative changes in radio sensitivity with increases in

5-BUdR incorporation have not been extensively related with changes in cellular mortality and chromosomal damage. This investigation, therefore, was concerned with an examination of the effects of incorporation of unifilar (growth for one generation) and bifilar (growth for two generations) amounts of TdR and its analogue 5-BUdR on cell survival and chromosomal damage in synchronised CHO cell populations.

In essence, the investigation attempted to determine the following:

(a) Whether the nature of either of the radiation responses was affected by the method of cell synchrony.

(b) The extent to which the two responses are correlated and whether the degree of correlation is influenced by cell cycle phase.

(c) To what extent the two responses are affected by unifilar labelling with TdR and 5-BUdR.

(d) The extent to which they are affected by bifilar labelling with TdR and 5-BUdR.

(e) The effect of the various treatments on cell cycle progression.

LITERATURE REVIEW

The generation cycle of dividing cells, first described in the bean, *Vicia faba*, root-tip (Howard and Pelc 1953), consists of the pre- and post-synthetic gaps, G_1 and G_2, the DNA synthetic period S and the mitotic phase M. This terminology is also characteristic of cell cycle analyses of mammalian cells cultured *in vitro* (Lajtha *et al.* 1954).

Cell Cycle Analysis

The doubling time (generation cycle) of mammalian cells grown *in vitro* is not the same for all cells of a given line. A population of cells exhibits values distributed over an average which is constant for a specific culture condition. The many mammalian cell lines that are now amenable to *in vitro* culture vary, in generation time, from 8 hrs to 30 hrs as well as in the duration of each cyclic phase (Sinclair 1968). Several techniques enable an assessment of the duration of these cyclic phases. One of the earliest methods ("pulse chase") involved the scoring of labelled *vs.* non-labelled mitotic cells harvested from an asynchronously growing culture subjected to a brief pulse of tritiated thymidine (Dewey and Humphrey 1962, Howard and Pelc 1953, Lajtha *et al.* 1958, and others). Dendy and Cleaver (1964) combined autoradiography with microspectrophotometry to characterise the relative duration of each phase. The technique of Puck and Steffen (1963), which involves the lengthy and tedious microscopic analysis of autoradiographs, determines the cell cycle by counting labelled *vs.* non-labelled metaphases in an asynchronous population treated simultaneously with colchicine and tritiated thymidine. A recent development in the characterisation of duration of cyclic phases involves the utilisation of synchronous cell populations. The technique takes advantage of the specific

uptake of tritiated thymidine into DNA, traced by liquid scintillation spectrometry, thereby eliminating the tedious analysis of autoradiographs (Miller *et al.* 1972). The cell cycle for a CHO strain (CCL-61) as determined by this method was in reasonable agreement with that determined by the autoradiographic method for the same strain (Miller 1971).

An understanding of the nature and duration of the cyclic phases has led to techniques being devised for synchronisation of cell populations.

Synchronisation Procedures

Several techniques are now available whereby cells can be synchronised in a specific phase of the cycle. Some of the procedures rely on chemicals for the interruption of specific cellular processes while others utilise distinct physical properties of cells for the induction of synchrony. A good review of the various methods employed in the synchronisation of cell populations has been presented in a recent symposium (Petersen *et al.* 1969).

Synchrony is often induced in the M phase, resulting in a tightly synchronised population of cells, since it is the shortest phase. This is usually accomplished either by the use of compounds known as mitotic inhibitors or poisons such as colcemid (Stubblefield and Klevecz 1965) and some vinca-alkaloids (Madoc-Jones and Mauro 1968) or by detachment from the growing surface through gentle shaking (Terasima and Tolmach 1963).

Colcemid Synchrony. Colcemid prevents the successful assemblage and subsequent function of the mitotic spindle, thereby resulting in the arrest of cells in metaphase. The arrested cells can be readily detached by gentle shaking and inhibition can then be reversed by centrifugation and resuspension in fresh, colcemid-free medium (Stubblefield and Klevecz 1965). Cells thus treated resume mitosis and traverse the subsequent cycle after a brief

delay (Puck and Steffen 1963, Stubblefield *et al.* 1967, and others). The cell population resulting from such a procedure is a relatively pure (85 to 95 percent) metaphase one. There is disagreement, however, as to whether cells thus synchronised behave predictably in subsequent cycles after synchrony. Ultrastructure studies (Brinkley and Stubblefield 1966, Brinkley *et al.* 1967) reveal that colcemid-treated cells form a mitotic apparatus of normal appearance after reversal. Some other studies also indicate that the kinetics of DNA, RNA and protein synthesis are unaffected by brief colcemid treatment (Stubblefield *et al.* 1967). Wagner and Roizman (1968), however, report an inhibition of RNA synthesis and Erbe *et al.* (1969) find indications of elevated RNase and DPNase activities. Robbins and Gonatos (1964), furthermore, report cytological changes associated with the Golgi apparatus. Cox and Puck (1969) and Kato and Yoshida (1970) find an increased frequency of chromosomal nondisjunction, dependent on the duration of colcemid arrest.

Such disturbances have not been encountered in cells synchronised by the detachment method of Terasima and Tolmach (1963), for which the only disadvantageous feature established to date is its failure to produce efficient yields of cells in the division phases (Petersen *et al.* 1969).

Shaking synchrony. Axelrad and McCulloch (1958) observed that mitotic cells appeared rounded and detached and could be easily removed from a monolayer culture. This phenomenon was first utilised successfully by Terasima and Tolmach (1963) for the induction of synchrony. They collected the loose mitotic cells by bathing a monolayer culture in a gentle stream of pipetted medium. As a result of various suggested modifications to improve the yield of mitotic cells (Lindahl and Sörenby 1966, Robbins and Marcus 1964), loose mitotic cells are currently collected and maintained in a chilled (4°C) water bath. Under these conditions they do not show any

inclination to traverse the cell cycle and the progress of cellular events in populations thus synchronised is normal (Petersen *et al.* 1969).

Both methods of synchronisation yield cell populations that are ready for entry into the G_1 interval of the cell cycle. Other procedures yield cells synchronised for entry into the S or G_2 intervals. Induction of synchrony, regardless of the cyclic point at which cells are synchronised, enables biochemical exploration of the steps in the eucaryotic cell cycle (Mueller 1969). It also permits an investigation of cellular radiation responses, which are usually "age-dependent" (Sinclair and Morton 1963, Terasima and Tolmach 1963, and others).

Radiation Responses

Cell survival and chromosomal damage, two presumably related aspects of radiosensitivity have been frequently studied in synchronised cell populations after irradiation *in vitro*. Normally, the degree of correlation between these responses varies with cell lines, which differ in the duration of the cell cycle. Occasionally these responses differ within a cell line between investigations carried out in different laboratories, possibly because of differences in synchronising and culturing techniques (Sinclair 1968). Nonetheless, there is general agreement that most of the radiation responses are dependent upon the position of a cell within its generation cycle (Hahn and Bagshaw 1966, Hsu *et al.* 1962, Sinclair and Morton 1963, 1966, and others).

Cell survival or colony forming ability. Radiosensitivity, as measured by survival and clonal generation of irradiated cells varies from line to line. Cell lines, in which the G_1 phase is relatively short, show a cyclic response suggesting that M and G_2 are extremely sensitive while S is much

less so (Sinclair and Morton 1966). Cell lines with a relatively long G_1 show sensitivity in G_1 as well (Sinclair 1968). In CHO cells, maximum post-irradiation survival occurs during S (Dewey *et al.* 1970, Hsu *et al.* 1962, Sinclair 1968, and others). This suggests a possible connection between DNA synthesis and cellular mortality in irradiated cells. However, maximum survival is not necessarily coincident with the point in the synthetic period during which DNA synthesis is at a maximum. Therefore, survival, an "age-dependent response," must involve other cellular metabolic processes as well, in addition to DNA synthesis (Sinclair 1968).

The incorporation of 5-BUdR into DNA prior to irradiation increases the sensitivity of CHO cells to ionising radiations (Dewey and Humphrey 1965). The increase is much too great to be accounted for by an additive interaction of X-ray damage with 5-BUdR toxicity (Dewey *et al.* 1971). Cells pretreated with 5-BUdR for at least one cycle prior to synchronisation and subsequent irradiation, indicate a parallel effect of this chemical on cyclic response, wherein M is still the most sensitive phase (Dewey *et al.* 1971).

Thus radiosensitivity, as measured by a reduction in colony formation, is dependent upon the cyclic position which, in turn, is qualified by the biochemical state of cells during irradiation. Chromosomal damage has also been implicated in the primary cause of reproductive death in mammalian cells (Greenblatt 1961, Puck 1958, and others). But it is not as yet entirely clear how chromosome aberrations are manifested after irradiation.

Chromosomal damage. Both qualitatively and quantitatively, the responses of cells to irradiation, in terms of chromosomal damage, differ with the cyclic phase in which the cells are irradiated. The chromatid type of aberration is characteristic of cells irradiated during S while the chromo-

some type occurs in cells irradiated during M, early G_1 and late G_2 (Evans 1962, Wolff 1968, and others). Investigations with some synchronised CHO lines indicate that chromatid exchanges occur in cells actively synthesizing DNA (Yu and Sinclair 1967) while chromosome exchanges occur in cells in metaphase and G_1 (Dewey *et al.* 1970 and others). However, Yu and Sinclair (1967) claim that cells irradiated in G_1 are most radiosensitive in terms of overall chromosomal damage while Dewey *et al.* (1970, 1971) contend that irradiated metaphase cells are most radiosensitive. The results of investigations with other cellular systems (Sparrow *et al.* 1952, St. Amand 1956, and others) concur that chromosomes are most sensitive during metaphase. Cells pretreated with 5-BUdR for one cycle prior to irradiation incur qualitatively similar chromosomal damage with the notable exception that the frequency of chromatid exchanges is elevated in mitotic and G_1 irradiated cells (Dewey *et al.* 1971). Here once again, quantitatively, the results suggest that the mitotic phase is the most radiosensitive *(loc. cit.)*.

Although correlations between fractions of cells killed and fractions of cells that contain chromosomal aberrations after irradiation are reasonably good, the relationship between the two responses is not entirely clear. Differences in cyclic radiosensitivity for both cell survival and chromosomal damage are often quantitatively expressed in terms of dose modifying factors (DMF). A dose modifying factor for any specified time after synchrony (X hours) is defined as the ratio of the doses, at X hours and 0 hours respectively, producing a given amount of radiation damage. Observed similarity in DMF's for the two types of response lends strong support to the hypothesis that radiation-induced cell death results from chromosomal damage (Dewey *et al.* 1970). Investigations with a Chinese hamster line (Don cells) synchronised by shaking and by colcemid, indicate that the

DMF's are similar for both chromosomal damage and cellular lethality (Dewey *et al.* 1970). CHO cells pretreated with 5-BUdR for one cycle prior to synchrony and subsequent irradiation suggest that the DMF's for the two responses appear quite similar to, but lower than, those for cells not pretreated with the base analogue (Dewey *et al.* 1971).

MATERIALS AND METHODS

Cells of the Chinese hamster ovary *(Cricetulus griseus)*, strain CCL-61 were obtained from the American Type Culture Collection Repository, Rockville, Md. From these a sub-line was derived by repeated cloning and used in all the experiments. This sub-line contained a near-diploid karyotype with a modal number of 20 chromosomes.

Stock Cultures

The cells were routinely grown in McCoy's 5a (modified) medium supplemented with the following:

10% heat-inactivated calf serum

5% heat-inactivated fetal calf serum,

50,000 IU/litre penicillin,

50,000 µg/litre streptomycin,

50,000 µg/litre neomycin, and

2.2 g/litre sodium bicarbonate.

The above reagents were all obtained from Grand Island Biological Co. (Gibco), N.Y. Stock cultures were maintained in 75 cm^2 Falcon plastic flasks, incubated at 37°C in an atmosphere of 5 percent CO_2 in air. The cells were sub-cultured every 48 to 72 hours by trypsinisation at room temperature for 2 to 5 minutes in 0.5 percent trypsin (Schwarz Bioresearch) in sterile Hanks Balanced salt solution (HBSS). Stock cultures were examined periodically to ensure that the karyotype of the sub-line had not deviated from the modal number of 20 chromosomes.

TdR and 5-BUdR Treatments

McCoy's 5a medium as constituted above was further supplemented with 3.4 µg/ml TdR (TdR McCoy's) or 4.0 µg/ml 5-BUdR (5-BUdR McCoy's). Stock

solutions of TdR and 5-BUdR (both obtained from General Biochemicals) were made in sterile HBSS.

Unifilar labelling experiments. Stock cultures were sub-cultured and grown to 3/4 confluency in large glass prescription bottles containing McCoy's 5a medium. Eighteen to 20 hours prior to cell synchronisation, the medium was discarded and fresh McCoy's 5a medium containing 3.4 µg/ml TdR or 4.0 µg/ml 5-BUdR was added to the cultures. At the end of this period, the medium was again discarded and the cultures were rinsed with sterile HBSS, prewarmed to 37°C and prepared for either of the two methods of synchrony described below. The cells, while being synchronised, were collected in the appropriate medium, TdR McCoy's or 5-BUdR McCoy's. Once synchronised and ready for radiation response studies, the cells were replated in 25 cm^2 Falcon plastic flasks, containing McCoy's 5a medium.

Bifilar labelling experiments. Stock cultures were sub-cultured and grown to 1/2 confluency in glass bottles containing McCoy's 5a medium. Thirty-six to 40 hours prior to cell synchronisation the medium was discarded and fresh appropriate medium, TdR McCoy's or 5-BUdR McCoy's, was added to the cultures. After 36 to 40 hours, the medium was again discarded and cultures were rinsed with sterile HBSS prewarmed to 37°C and prepared for synchrony. Here again, while being synchronised, the cells were collected in medium containing the appropriate additional supplement. The cells upon synchronisation were replated in 25 cm^2 Falcon plastic flasks containing McCoy's 5a medium.

Methods of Cell Synchrony

Selection method of synchrony - mechanical shaking. Triplicate confluent cultures were rinsed with sterile, prewarmed (37°C) HBSS and immersed in fresh McCoy's 5a medium. For TdR and 5-BUdR studies, rinsed triplicate

confluent cultures were re-immersed in the appropriate medium, TdR or 5-BUdR McCoy's 5a. In all experiments involving this method of synchrony, the cultures were then shaken at 15 minute intervals over a period of three hours, for 20 seconds in a mechanical shaker (Eberbach, high speed 280 s.p. min). After each shaking the supernatant medium containing loose cells was decanted off and fresh medium was added. Supernatant from the first four shakings was discarded to ensure a reasonable degree of synchrony. Subsequent supernatants were collected and the suspended cells were maintained in mitosis in an ice-water bath at 4°C. The cells were then centrifuged at 2700 rpm for 7 minutes at 4°C (International PR-2 refrigerated centrifuge, head #284). The medium was decanted off and the synchronised cells were suspended in 5 to 10 ml of fresh, sterile, chilled McCoy's 5a medium.

Colcemid method of synchrony - chemical arrest. Triplicate confluent cultures, after being initially washed with sterile HBSS, were immersed in fresh, warm (37°C) McCoy's 5a medium containing 0.06 µg/ml colcemid in Hanks saline solution (Gibco). For TdR and 5-BUdR investigations, rinsed confluent cultures were re-immersed in medium containing the metaphase arrestor plus the appropriate supplement. Colcemid treatment was maintained for three hours after which time the metaphase cells were collected by shaking the cultures for 20 seconds in the mechanical shaker. The cells were then centrifuged at 2700 rpm for 7 minutes at 4°C. The supernatant was poured off entirely and the cells were resuspended in 5 to 10 ml of fresh, sterile, chilled McCoy's 5a medium.

Fresh suspensions of synchronised cells were held at 4°C to maintain synchrony until they were ready to be plated for radiation response studies, *viz.*, survival and/or chromosomal aberrations.

Radiation Responses

Survival (Colony forming ability). The cell concentration in each of the synchronised cell suspensions was determined with a Coulter counter. Each concentration was then adjusted to suit the treatment to which the cells were to be exposed, *viz.*, different numbers of cells were plated (approx. 40 cells/ml to approx. 11,000 cells/ml) in 25 cm^2 plastic flasks (4 ml/flask), depending on the radiation dose. The various samples were irradiated with doses ranging from 0 R to 1000 R, at specific times post-synchrony. One set of flasks (a) comprising triplicate samples per radiation dose, was irradiated immediately after collecting and plating the synchronised cells. This set of flasks (0 hours post-synchrony) was designated as the one in which the cells were in the mitotic phase. Three other triplicated sets treated in the same way were irradiated after specific periods of post-synchrony growth—(b) 2 to 3 hours (G_1), (c) 8 to 9 hours (S) and (d) 12 to 13 hours (late S - early G_2).

The time required to irradiate a complete set of 30 flasks for a given phase of the cell cycle was less than 20 minutes. A specific dose sample consisting of three flasks, for any given cyclic phase, was removed from the incubator only while being irradiated. This avoided subjecting the cells to fluctuations in temperature once they had been plated for survival response.

In addition to controls (0 R) and radiation dose samples (100 R to 1000 R), triplicate multiplicity flasks were plated for each system promptly upon synchronisation. These flasks were sacrificed 9 to 10 hours after synchrony to determine average multiplicity values ($\bar{N}$), *viz.*, the average number of cells involved in the formation of a single colony.

The controls and other radiation dose samples, unlike the multiplicity samples, were incubated at 37°C for 8 to 9 days in a humid atmosphere of 5 percent CO_2 in air. The medium was then discarded and the colonies were stained with crystal violet and counted. Average plating efficiency values (P.E.) were determined for each system from the triplicate control samples. From these, fractional survival values (f) for the other dose samples were calculated and corrected for multiplicity to obtain single cell survival values (S), according to the equation $f = 1 - (1 - S)^{\bar{N}}$ (Sinclair and Morton 1965). Plots of S *vs.* dose were constructed for each cyclic phase of the ten different systems.

Chromosomal aberrations. The cell concentration of each synchronised population was determined with a Coulter counter. The concentration was adjusted to approximately 3 - 5 x 10^5 plated cells per flask. The flasks were irradiated with doses ranging from 0 R (control) to 500 R at specific times (M, G_1, S and late S-early G_2) after synchrony. The time required to irradiate a complete set of five flasks for each prescribed phase of the cell cycle was less than seven minutes. Incubation, at 37°C in a humid atmosphere of 5 percent CO_2 in air, for the various samples, was interrupted only during irradiation. At 20 to 21 hours after synchrony, 0.06 µg/ml colcemid, constituted in HBSS (Gibco), was added to all samples of each cyclic phase. Subsequent to a 4-hour treatment for the accumulation of first division post-irradiation metaphases, the samples were harvested for chromosome preparations. Metaphase cells were swollen in hypotonic salt solution (1 percent sodium citrate in deionised water) at 37°C for 15 minutes and fixed in cold (4°C) 3:1 v/v methanol-glacial acetic acid for two hours. Chromosome preparations were made by spreading a drop of the fixed suspension on slides predipped in acetic acid and drying over a flame. The slides were

stained in 2 percent aceto-orcein, mounted in Euparal and examined for aberrations under a 90X oil-immersion, phase-contrast objective. From 30 to 50 cells were examined per dose sample for each cyclic phase. Aberrations were scored as chromatid deletions and/or gaps (1 break), isochromatid deletions (1 break), chromatid exchanges (2 breaks) and chromosome exchanges, *viz.*, rings, dicentrics (2 breaks) and tricentrics (4 breaks). Controls were scored for aberrations for the calculation of the average number of breaks per unirradiated cell. This value was subtracted from the average number of breaks per irradiated cell for each of the other doses to obtain the final values for a plot of dose *vs.* number of induced breaks per cell.

Irradiation procedure. Irradiation was carried out under normal lighting conditions and at room temperature. The standard conditions of radiation were: 300 kVa, 20 mA, dose rate 350 R/min, delivered at a distance of 19 cm from a Model Philips machine with a HVL of 5 mm Aluminium.

Estimation of Degree of Synchrony

Although an estimation of the mitotic index of a cell suspension is not an accurate measure of the degree of synchrony, the mitotic index of each synchronised cell population was scored immediately after synchronisation to determine the degree of synchrony at time zero. This was accomplished by subjecting a 2 ml aliquot of synchronised cells to the procedure for chromosome preparation as described on page 15. From 300 to 500 cells for each suspension were counted for an estimation of the mitotic index and, therefore, the initial degree of synchrony.

Cell Progression Profile

The behaviour of cells (cell progression) through at least one

generation after synchrony was investigated for each system. This was accomplished by studying the hourly uptake of tritiated thymidine (^{3}H TdR) by synchronised cells resuspended in preconditioned vials containing labelled McCoy's 5a medium (0.5 µCi/ml - ^{3}H TdR s.a. 6.7 Ci/mM). The procedure followed was similar to that of Miller *et al.* (1972). Pre-treatment of cells with TdR and 5-BUdR was as described on pages 11 and 12 and the methods of synchrony employed were as described on pages 12 and 13.

TdR and 5-BUdR Incorporation

The extent of incorporation of both exogenous thymidine and its analogue 5-BUdR was assessed in both the unifilar and bifilar labelling experiments. This was achieved by growing stock cultures as described on pages 11 and 12 except that for one and two generations of growth prior to synchronisation, the cells were grown in McCoy's 5a medium supplemented with either ^{3}H TdR (0.5 µCi/ml - s.a. 6.7. Ci/mM) or ^{3}H 5-BUdR (0.5 µCi/ml - s.a. 12.7 Ci/M). For unifilar labelling experiments the cultures were trypsinised within 20 hrs of adding the labelled supplement (^{3}H TdR or ^{3}H 5-BUdR). The cultures were trypsinised within 40 hrs for assessment of bifilar incorporation. Subsequent to trypsinisation the cells were centrifuged at 800 r.p.m. for 5 min. The supernatant was discarded and the cells were treated with 0·5 N NaOH to hydrolyse the DNA. The DNA was then acid precipitated with 5% cold TCA and collected on glass filters. These glass filters were prepared for liquid scintillation spectrophotometry according to the method of Miller *et al.* (1972). The extent of incorporation of TdR or 5-BUdR was then determined according to the method of Dewey and Humphrey (1965).

Statistical Analysis

Variation in the radiation response chromosomal damage, as caused by the various systems, was tested statistically utilising Student's 't' test. Correlation coefficients 'r' were calculated to assess the relationship between chromosomal damage and cell survival, in each of the cyclic phases of the different systems. Finally, the degree of radiosensitivity of each cyclic phase, together with the effects of the various treatments on chromosomal damage, were evaluated on the basis of analysis of variance and calculation of the least significant difference (L.S.D.).

RESULTS

The raw data for the various observations are tabulated in the Appendix.

A. Cell Progression Profiles

The different cell progressions are depicted in Figures 1 to 5. Figure 1a represents the cell cycle for a CHO cell strain, CCL-61, as determined by Miller (1971). Figure 1b represents the cycle for the same strain, utilising the colcemid method of synchrony. The two profiles indicate that, apart from a brief initial delay of about 1.5 to 2 hours in the initiation of DNA synthesis in the colcemid system, the durations of the cell cycle are similar (16 to 17 hrs.). Figures 2 to 5 represent the progression profiles for cells subjected to unifilar and bifilar treatments of TdR and its analogue 5-BUdR. The 5-BUdR treatment profiles (Figures 2 and 3) for both methods of synchrony suggest a delay in the initiation of DNA synthesis comparable to the delay indicated in the colcemid method (Figure 1b). The unifilar and bifilar TdR shaking profiles (Figures 4a and 5a) show no deviation from the profile for normal shaking (Figure 1a). Similarly, the TdR colcemid profiles (Figures 4b and 5b) exhibit no deviation from that for normal colcemid (Figure 1b). The durations of the four cyclic phases as characterised by Miller (1971) were: $M+G_1=4.60$ hrs., $S=9.60$ hrs. and $G_2=1.85$ hrs., while the durations as characterised by the colcemid method are: $M+G_1=6.5$ hrs., $S=8.0$ hrs. and $G_2=1$ hr. approximately.

B. Chromosomal Damage

This response was quantitated in terms of chromosomal aberrations

as described in Materials and Methods. The summarised data for aberrations (breaks) per cell for the various systems are presented in Table 1. The aberrations per cell *vs.* dose responses, for each cyclic phase of the different systems, were fitted by eye and are represented in Figures 6 (a,b) to 10 (a,b). The graphs reveal that an increase in radiation dose is accompanied by an increase in chromosomal damage. The nature of the response in terms of the shape of the curve for the M phase in all the systems is linear. The three other phases, G_1, S and late S-early G_2, demonstrate a curvi-linear response to increasing radiation.

The qualitative nature of chromosomal damage in terms of types of aberrations observed for the different phases of the various systems is depicted in Tables i-x in the Appendix. The observations suggest that chromosome type aberrations are prominent during mitosis, while chromatid type aberrations increase in frequency as the cell traverses the cycle. For unifilar and bifilar 5-BUdR systems, an increase in the total number of chromatid aberrations during mitosis is indicated. This increase is slightly greater for the colcemid method of synchrony for both 5-BUdR treatments. A similar trend is not evident in the TdR treatments (Tables iii - x in Appendix).

C. Cell Survival

Single cell surviving fractions (S) calculated according to the equation $f = 1 - (1-S)^{\bar{N}}$, for each phase of the various systems, are tabulated in the Appendix (Tables xi - xv). The semilogrithmic plots of percent surviving fraction *vs.* radiation dose were fitted in by eye and are represented by Figures 11 to 20. It is apparent from the graphs that an increase in radiation dose is accompanied by a reduction in cell survival.

The linear shape of the response curve is characteristic of the M phase in all the systems while the other cyclic phases indicate a curvi-linear response. Unifilar and bifilar treatments with 5-BUdR for both methods of synchrony reveal an enhanced reduction in survival in all the cyclic phases (Figures 13 to 16). Treatments of identical duration with TdR do not suggest a similar reduction in survival (Figures 17 to 20).

D. Statistical Evaluations

Table 2 shows the coefficients of correlation (r) for each of the cyclic phases of the different systems. The negative values of 'r' indicate that an increase in chromosomal damage is accompanied by a corresponding decrease in cell survival. Table 3 represents a comparison of chromosomal damage, caused by the various systems, on the basis of Student's 't' test. The 't' values reveal that the extent of chromosomal damage in systems utilising 5-BUdR is significantly different from that in other systems. An analysis of variance followed by calculation of L.S.D. also suggests that systems incorporating 5-BUdR are more sensitising in terms of chromosomal damage than TdR and normal systems. Systems incorporating TdR are not significantly different for the response than the normals. The above test also indicates that the mitotic phase is the most sensitive in terms of chromosomal aberrations, for all the systems. The results are presented below in terms of the means and their L.S.D.

a. Normal and unifilar systems (L.S.D. = 2.01)

Phases	M	Late S-early G_2	G_1	S
Means	18.9	13.5	13.4	13.1

Systems	E	F	A	C	B	D
Means	24.3	17.2	13.2	12.1	11.1	10.4

b. Normal and bifilar systems (L.S.D. = 1.90)

Phases	M	Late S-early G_2	G_1	S
Means	18.9	14.7	14.1	13.8

Systems	I	J	A	B	G	H
Means	25.4	23.0	13.2	11.1	10.3	8.4

c. Unifilar and bifilar systems (L.S.D. = 3.85)

Phases	M	Late S-early G_2	S	G_1
Means	20.4	15.6	15.1	14.5

Systems	I	E	J	F	C	D	G	H
Means	25.4	24.3	23.0	17.2	12.1	10.4	10.3	8.4

Note: Any two means underscored by the same line are not significantly different (difference between means is less than L.S.D.) and vice versa.

Legend: A—Normal Colcemid; B—Normal Shaking; C—Unifilar TdR Colcemid; D—Unifilar TdR Shaking; E—Unifilar 5-BUdR Colcemid; F—Unifilar 5-BUdR Shaking; G—Bifilar TdR Colcemid; H—Bifilar TdR Shaking; I—Bifilar 5-BUdR Colcemid; J—Bifilar 5-BUdr Shaking.

Table 4 shows the doses necessary in the different cyclic phases to produce an arbitrary level of chromosomal damage and reduce cell survival to an arbitrary level. The shift in doses further indicates that the mitotic phase is the most radiosensitive for both radiation responses.

E. Calculation of TdR and 5-BUdR Incorporation

A typical calculation for analogue replacement is shown in the Appendix. The values obtained were as follows:

	Exogenous TdR	5-BUdR
Unifilar labelling	19%	13%
Bifilar labelling	26%	17%

Table 1

Summarised data for chromosomal aberrations.

Dose (R)	Normal Treatments				Unifilar Treatments								Bifilar Treatments								Cell Cycle Phase
	Normal Colcemid		Normal Shaking		TdR Colcemid		TdR Shaking		5-BUdR Colcemid		5-BUdR Shaking		TdR Colcemid		TdR Shaking		5-BUdR Colcemid		5-BUdR Shaking		
	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	No.of cells	Breaks per cell	
Control (OR)	66	0.21	45	0.27	47	0.38	62	0.19	65	0.63	57	0.63	50	0.20	60	0.20	40	1.00	52	0.96	Mitosis (0 hrs)
100	43	1.44	67	0.80	50	1.48	50	0.83	48	1.74	47	1.03	40	0.62	50	0.36	40	2.65	50	2.26	
200	33	2.88	44	1.84	41	1.91	51	1.59	45	3.93	51	2.60	45	1.53	47	0.95	35	4.60	43	4.13	
300	35	3.27	35	2.59	--	3.20*	60	1.96	36	7.50	53	3.71	40	2.62	50	1.92	30	6.66	31	5.49	
400	35	3.64	41	3.43	42	4.19	51	3.53	--	9.00*	45	4.21	35	3.08	42	2.18	30	9.60	35	6.75	
500	30	5.62	34	4.43	32	5.62	40	4.26	20	11.57	31	7.27	38	4.06	45	2.47	30	9.10	32	6.95	
100	47	0.83	41	0.87	50	0.64	55	0.54	50	1.23	56	1.05	38	0.46	50	0.42	35	1.74	50	1.82	G_1 phase (2-3 hrs)
200	44	1.81	37	1.08	--	1.35*	55	1.15	43	2.11	51	2.07	37	1.26	50	1.08	33	3.21	50	2.92	
300	45	2.72	34	1.91	44	1.78	41	1.98	38	3.60	47	2.75	33	2.01	50	1.22	31	4.42	50	4.64	
400	35	3.36	39	2.91	46	2.27	52	2.13	40	6.44	--	4.00*	39	2.36	43	1.54	35	4.51	40	5.44	
500	32	5.00	44	3.59	45	3.66	42	3.14	37	6.83	40	4.99	38	2.96	44	2.30	--	7.55*	40	6.06	
100	46	0.61	40	0.68	56	0.49	38	0.47	58	0.97	50	1.07	40	0.45	50	0.42	30	1.80	48	1.33	S phase (8-9 hrs)
200	42	0.95	37	1.21	52	1.35	40	1.18	46	2.46	--	2.25*	40	1.42	50	1.22	35	3.02	40	2.54	
300	61	1.90	35	1.90	46	2.23	40	2.11	38	3.60	--	3.25*	38	1.80	50	2.18	33	4.39	--	4.00*	
400	36	2.01	35	2.64	45	2.86	--	2.35*	35	6.65	50	4.69	39	2.82	40	2.27	30	5.20	35	5.18	
500	43	4.01	47	3.82	53	3.32	30	3.01	36	6.60	50	5.87	35	3.22	40	2.95	--	7.10	30	6.74	
100	41	0.98	45	0.61	48	0.72	40	0.76	46	1.13	47	1.13	40	0.50	50	0.54	30	1.60	50	1.80	late S—early G_2 (12-13 hrs)
200	50	1.21	40	1.00	36	1.45	55	1.31	46	2.56	50	1.89	--	1.20*	45	1.26	30	3.20	50	3.04	
300	43	2.37	49	1.77	42	2.00	50	2.37	41	4.76	44	3.00	38	2.27	49	2.04	--	4.15*	40	4.79	
400	31	3.11	31	2.53	45	2.64	40	2.88	35	6.05	43	3.69	35	2.65	46	2.47	30	6.10	38	5.90	
500	--	4.35*	50	3.67	46	3.57	43	3.40	31	5.82	40	5.84	35	3.48	41	3.11	30	7.06	37	6.44	

*Values obtained from graphs

Table 2

Correlation coefficients 'r' to show the relationship between aberrations and survival in different cyclic phases of different systems.

Systems ↓ \ Cyclic Phases	Mitosis (0 hrs)	G_1 (2-3 hrs)	S (8-9 hrs)	Late S—Early G_2 (12-13 hrs)
Normal Colcemid	-0.87*	-0.91*	-0.85*	-0.94**
Normal Shaking	-0.97**	-0.93**	-0.97**	-0.96**
Unifilar TdR Colcemid	-0.87*	-0.88*	-0.98**	-0.96**
Unifilar TdR Shaking	-0.89*	-0.96**	-0.97**	-0.99**
Unifilar 5-BUdR Colcemid	-0.91*	-0.83*	-0.85*	-0.94**
Unifilar 5-BUdR Shaking	-0.85*	-0.88*	-0.92*	-0.84*
Bifilar TdR Colcemid	-0.98**	-0.98**	-0.98**	-0.98**
Bifilar TdR Shaking	-1.00**	-0.94**	-0.98**	-0.99**
Bifilar 5-BUdR Colcemid	-0.81	-0.95**	-0.93**	-0.83
Bifilar 5-BUdR Shaking	-0.88*	-0.90*	-0.86*	-0.88*

* - significant at 5 percent
** - significant at 1 percent

Table 3

A Comparison of Chromosomal damage, as caused by various treatments, on the basis of Student's t-test

t values → / ← System	A	B	C	D	E	F	G	H	I	J
B	0.760	--	--	--	--	--	--	--	--	--
C	0.376	0.403	--	--	--	--	--	--	--	--
D	1.093	0.361	0.739	--	--	--	--	--	--	--
E	2.675*	3.245**	2.918**	3.464**	--	--	--	--	--	--
F	1.400	2.093*	1.698	2.366*	1.419	--	--	--	--	--
G	0.978	0.229	0.620	0.135	3.393**	2.277*	--	--	--	--
H	2.140*	1.501	1.789	1.127	4.115**	3.221**	1.287	--	--	--
I	3.032**	3.664**	3.298**	3.901**	0.151	1.677	3.825**	4.626**	--	--
J	3.356**	4.195**	3.691**	4.504**	0.064	1.665	4.418**	5.509**	0.245	--

* - significant at 5%
** - significant at 1%

Legend

A - Normal Colcemid
B - Normal Shaking
C - Unifilar TdR Colcemid
D - Unifilar TdR Shaking
E - Unifilar 5-BUdR Colcemid
F - Unifilar 5-BUdR Shaking
G - Bifilar TdR Colcemid
H - Bifilar TdR Shaking
I - Bifilar 5-BUdR Colcemid
J - Bifilar 5-BUdR Shaking

Table 4

A comparison of doses necessary to produce an arbitrary level of chromosomal damage and reduce cell survival to an arbitrary level.

System ↓	Dose (R) necessary to produce 5 aberrations/ cell in the different cyclic phases				Dose (R) necessary to reduce cell survival to 1% in the different cyclic phases			
	Mitosis (0 hrs)	G_1 (2-3 hrs)	S (8-9 hrs)	late S-early G_2	Mitosis (0 hrs)	G_1 (2-3 hrs)	S (8-9 hrs)	late S-early G_2
A	435 R	515 R	620 R	580 R	570 R	620 R	690 R	660 R
B	580 R	650 R	675 R	695 R	540 R	680 R	700 R	720 R
C	490 R	710 R	740 R	660 R	690 R	785 R	740 R	840 R
D	595 R	680 R	750 R	645 R	765 R	810 R	-	860 R
E	230 R	390 R	360 R	330 R	440 R	460 R	480 R	500 R
F	400 R	510 R	450 R	490 R	525 R	575 R	-	600 R
G	630 R	790 R	725 R	690 R	640 R	690 R	720 R	760 R
H	730 R	930 R	820 R	765 R	705 R	765 R	810 R	775 R
I	210 R	345 R	375 R	325 R	340 R	370 R	405 R	320 R
J	275 R	365 R	410 R	340 R	370 R	410 R	390 R	430 R

Legend as in Table 3.

Figure 1. Profiles showing the uptake of ^{3}H TdR by normal CHO cells synchronised by (a) shaking (obtained from G. Miller 1971) represented by open circles, and (b) colcemid represented by closed circles.

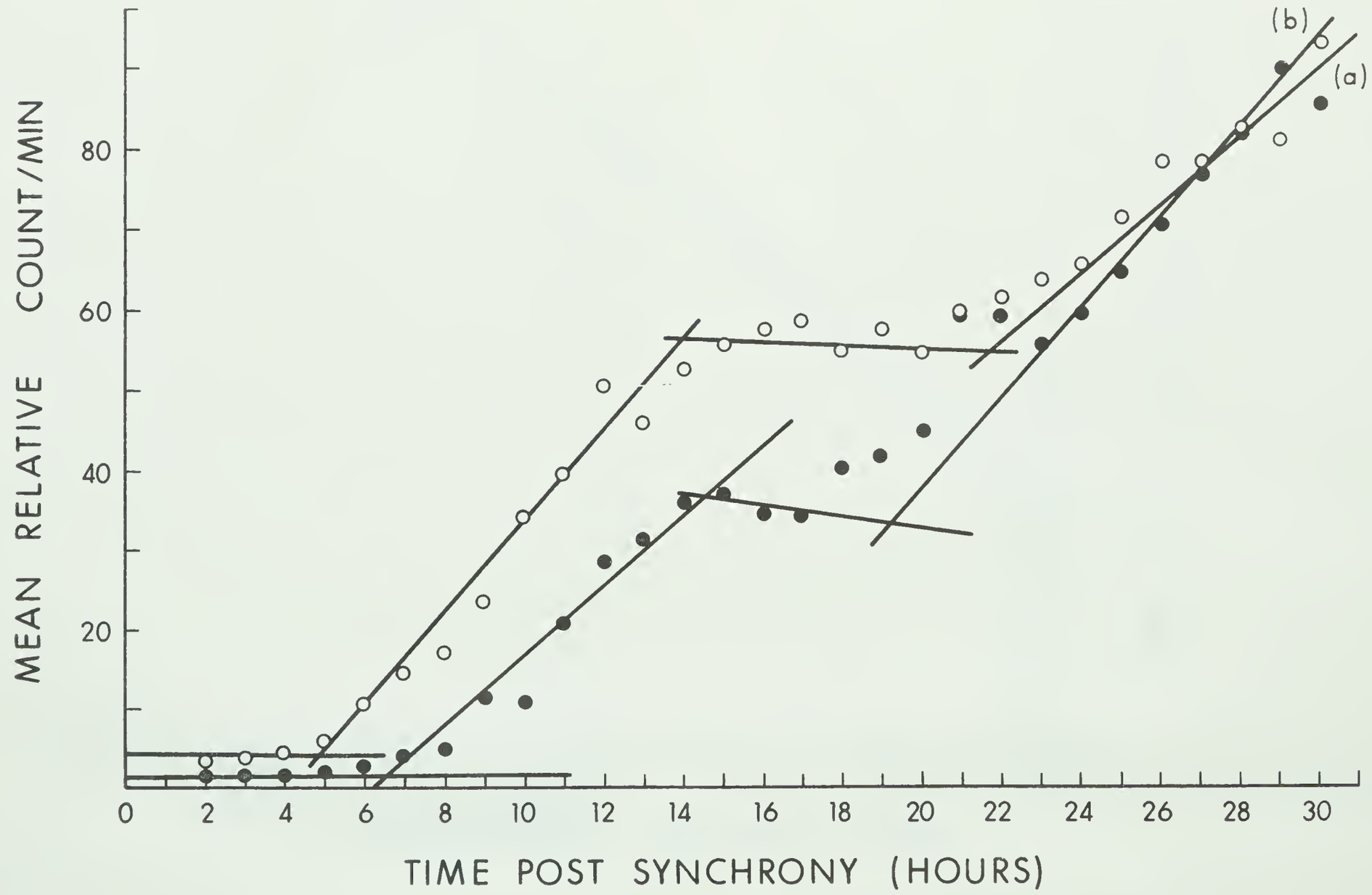

MEAN RELATIVE COUNT/MIN
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
TIME POST SYNCHRONY (HOURS)
(b)
(a)

Figure 2. Profiles showing the uptake of ^{3}H TdR by unifilar 5-BUdR labelled CHO cells synchronised by (a) shaking and (b) colcemid. See Figure 1 for symbols.

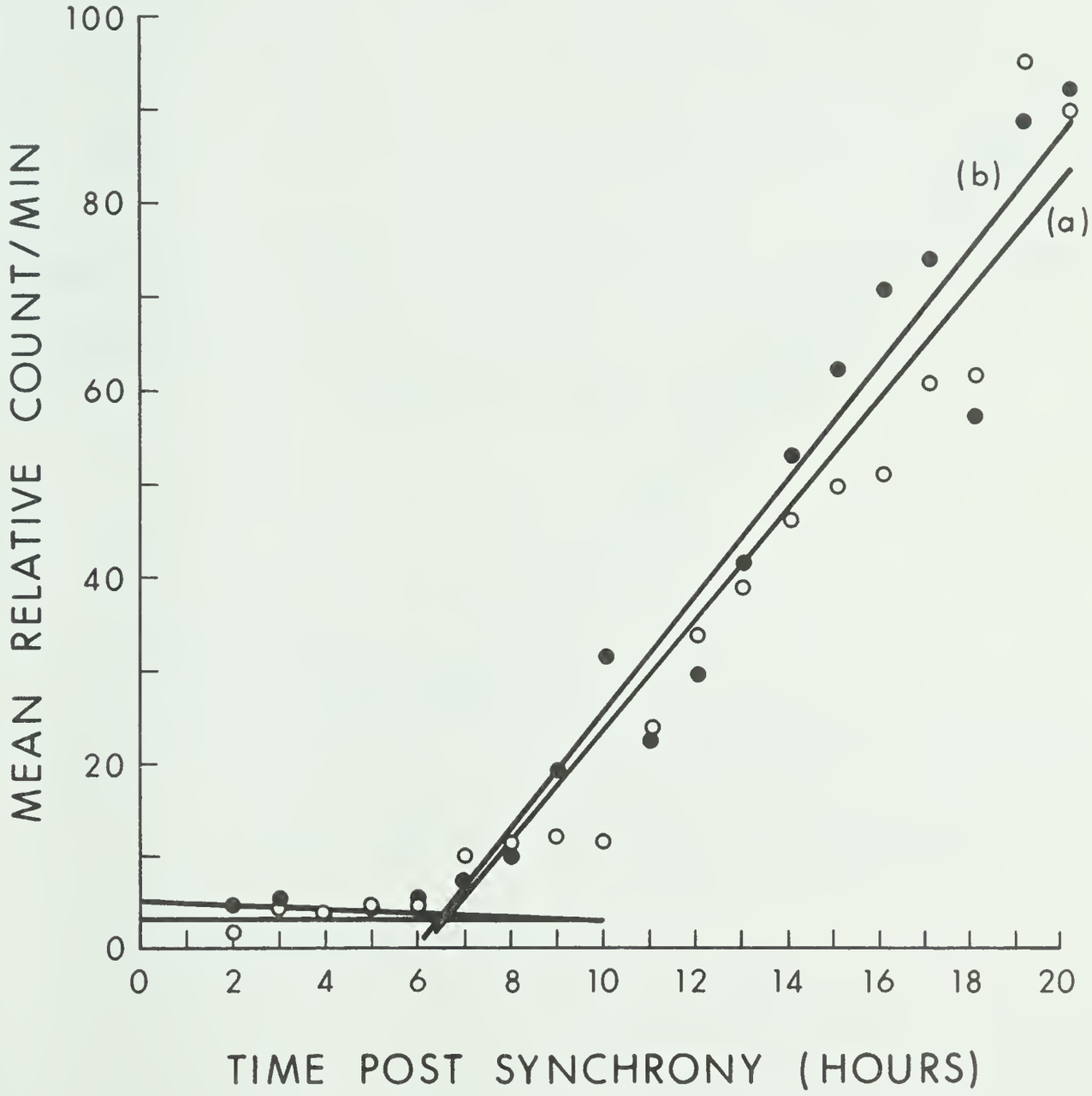

100
80
60
40
20
0
MEAN RELATIVE COUNT/MIN
0
2
4
6
8
10
12
14
16
18
20
TIME POST SYNCHRONY (HOURS)
(b)
(a)

Figure 3. Profiles showing the uptake of ^{3}H TdR by bifilar 5-BUdR labelled CHO cells synchronised by (a) shaking and (b) colcemid. See Figure 1 for symbols.

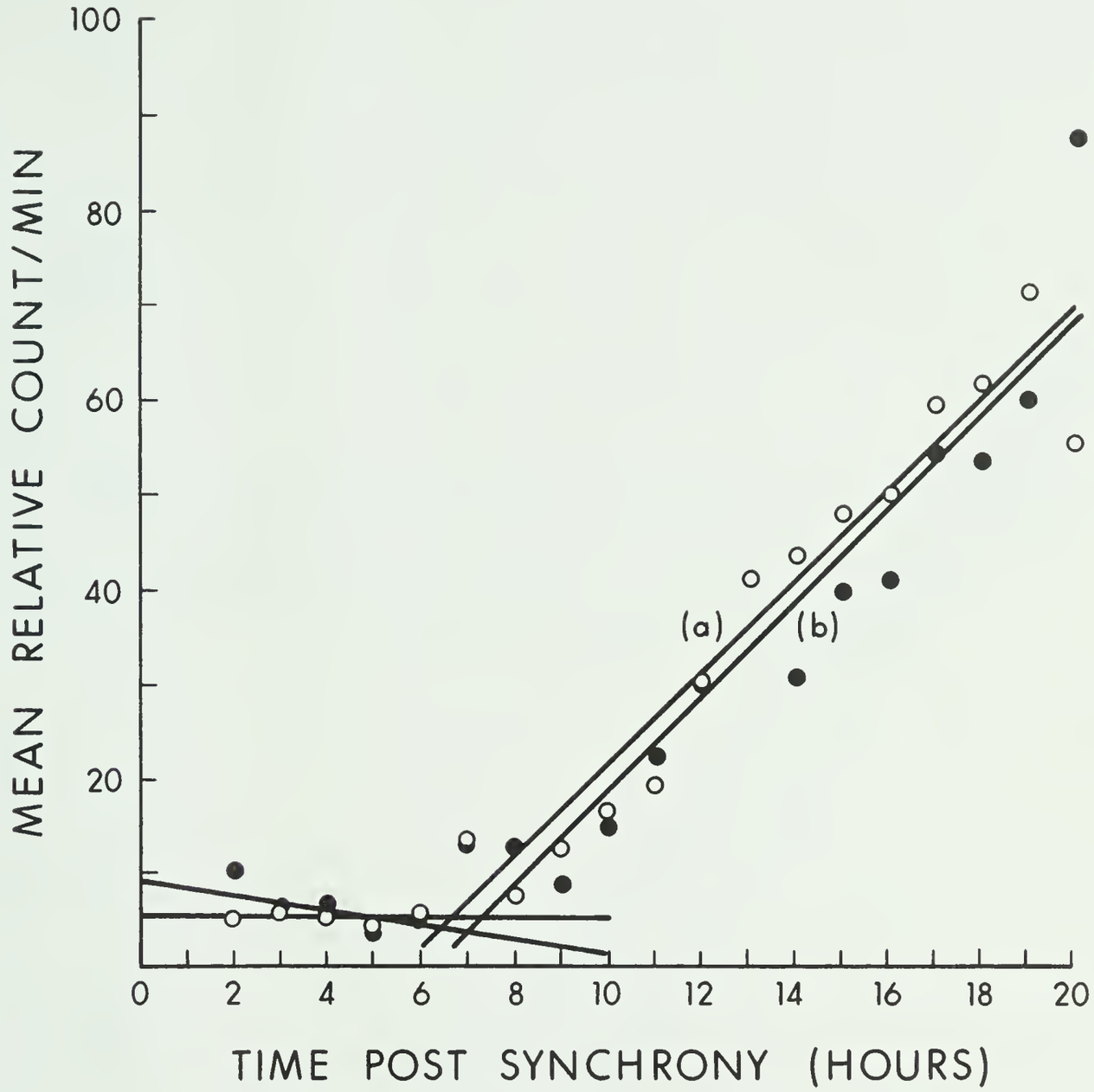

100
80
60
40
20
MEAN RELATIVE COUNT/MIN
(a)
(b)
0
2
4
6
8
10
12
14
16
18
20
TIME POST SYNCHRONY (HOURS)

Figure 4. Profiles showing the uptake of ^{3}H TdR by unifilar TdR labelled CHO cells synchronised by (a) shaking and (b) colcemid. See Figure 1 for symbols.

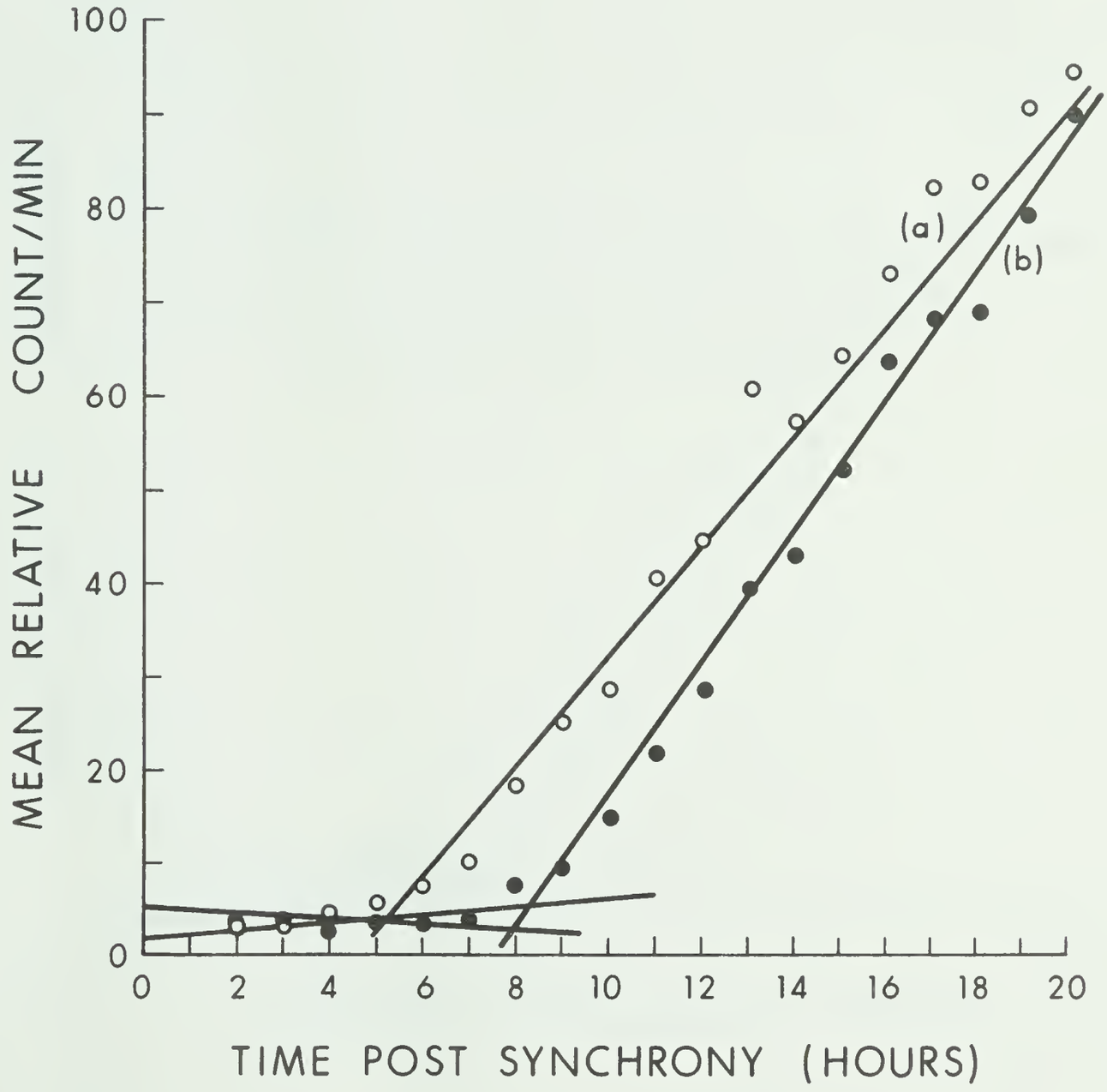

MEAN RELATIVE COUNT/MIN
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
TIME POST SYNCHRONY (HOURS)
(a)
(b)

Figure 5. Profiles showing the uptake of ^{3}H TdR by bifilar TdR labelled CHO cells synchronised by (a) shaking and (b) colcemid. See Figure 1 for symbols.

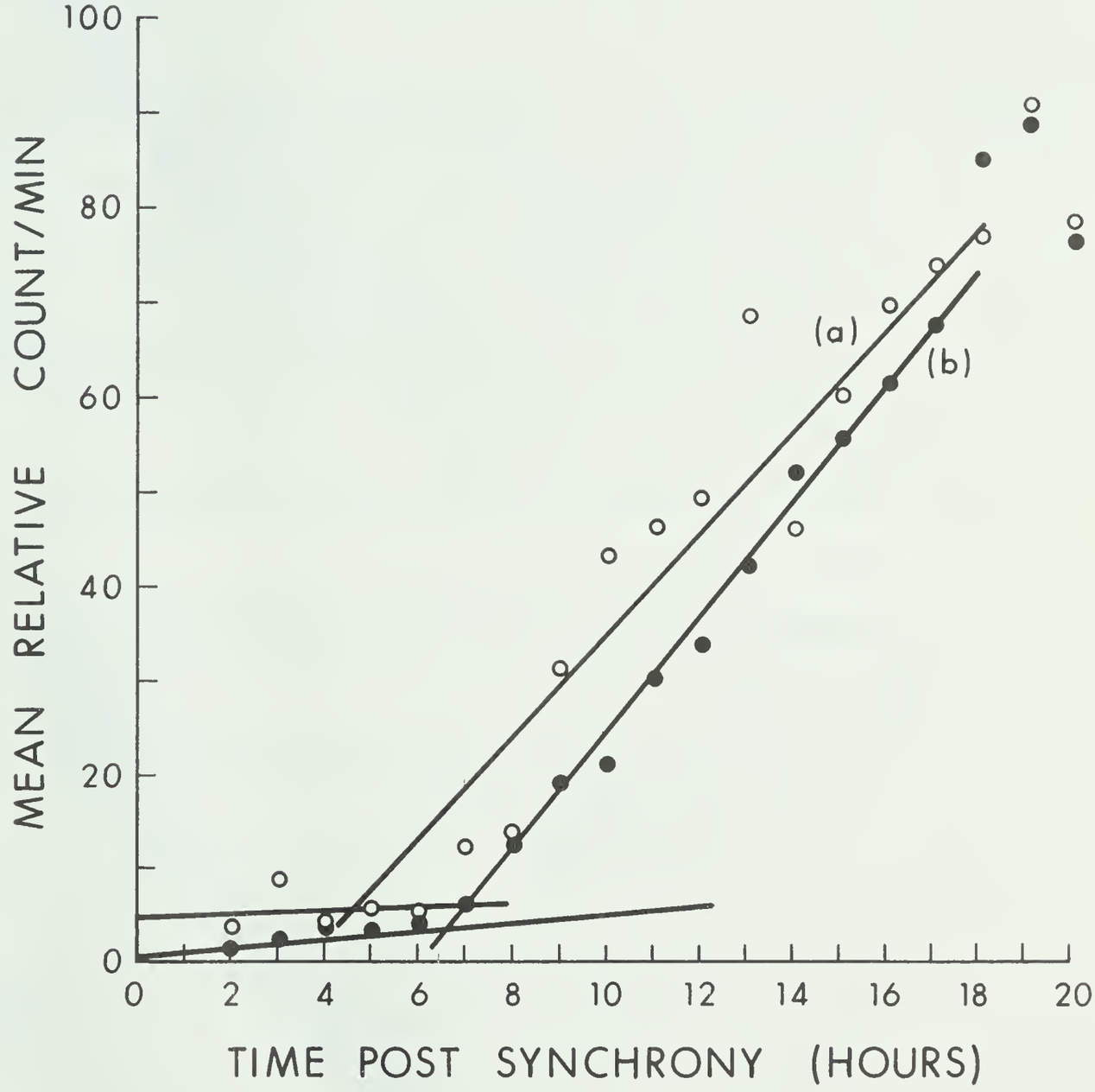

100
80
60
40
20
0
MEAN RELATIVE COUNT/MIN
0
2
4
6
8
10
12
14
16
18
20
TIME POST SYNCHRONY (HOURS)
(a)
(b)

Figure 6. Dose-effect curves for chromosomal damage in normal CHO cells irradiated at different times post-synchrony.

a) Cells synchronised in mitosis with colcemid.

b) Cells synchronised in mitosis by shaking.

Squares and solid lines indicate irradiation at mitosis (0 hrs).

Closed circles and solid lines indicate irradiation at G_1(2 - 3 hrs).

Triangles and dotted lines indicate irradiation at S (8 - 9 hrs).

Open circles and dotted lines indicate irradiation at late S-early G_2 (12 - 13 hrs).

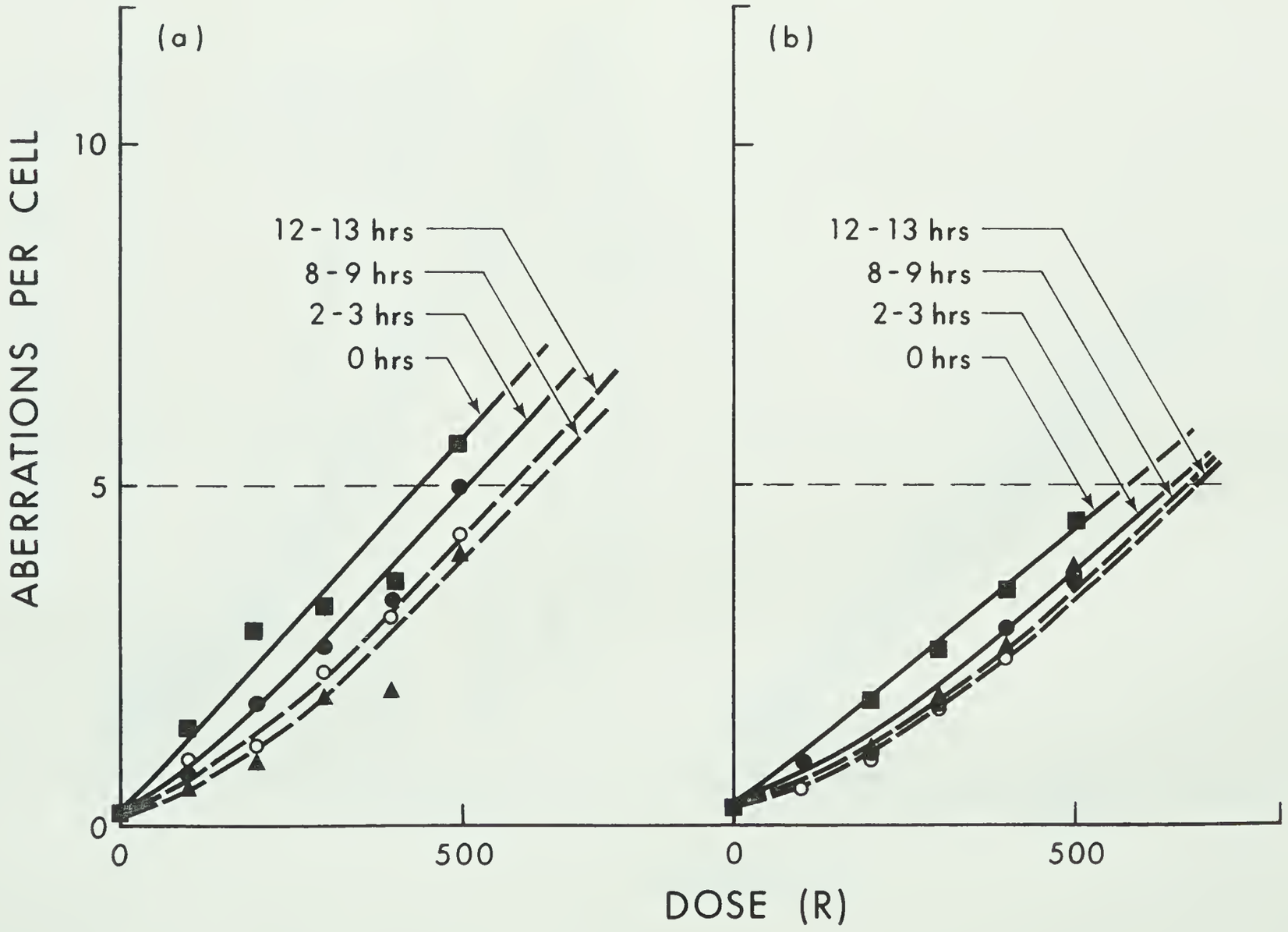

(a)
(b)
ABERRATIONS PER CELL
10
5
0
12 - 13 hrs
8 - 9 hrs
2 - 3 hrs
0 hrs
0
500
DOSE (R)

Figure 7. Dose-effect curves for chromosomal damage in unifilar 5-BUdR labelled CHO cells irradiated at different times post-synchrony.

a) Cells synchronised in mitosis with colcemid.

b) Cells synchronised in mitosis by shaking.

For symbols refer to legend in Figure 6.

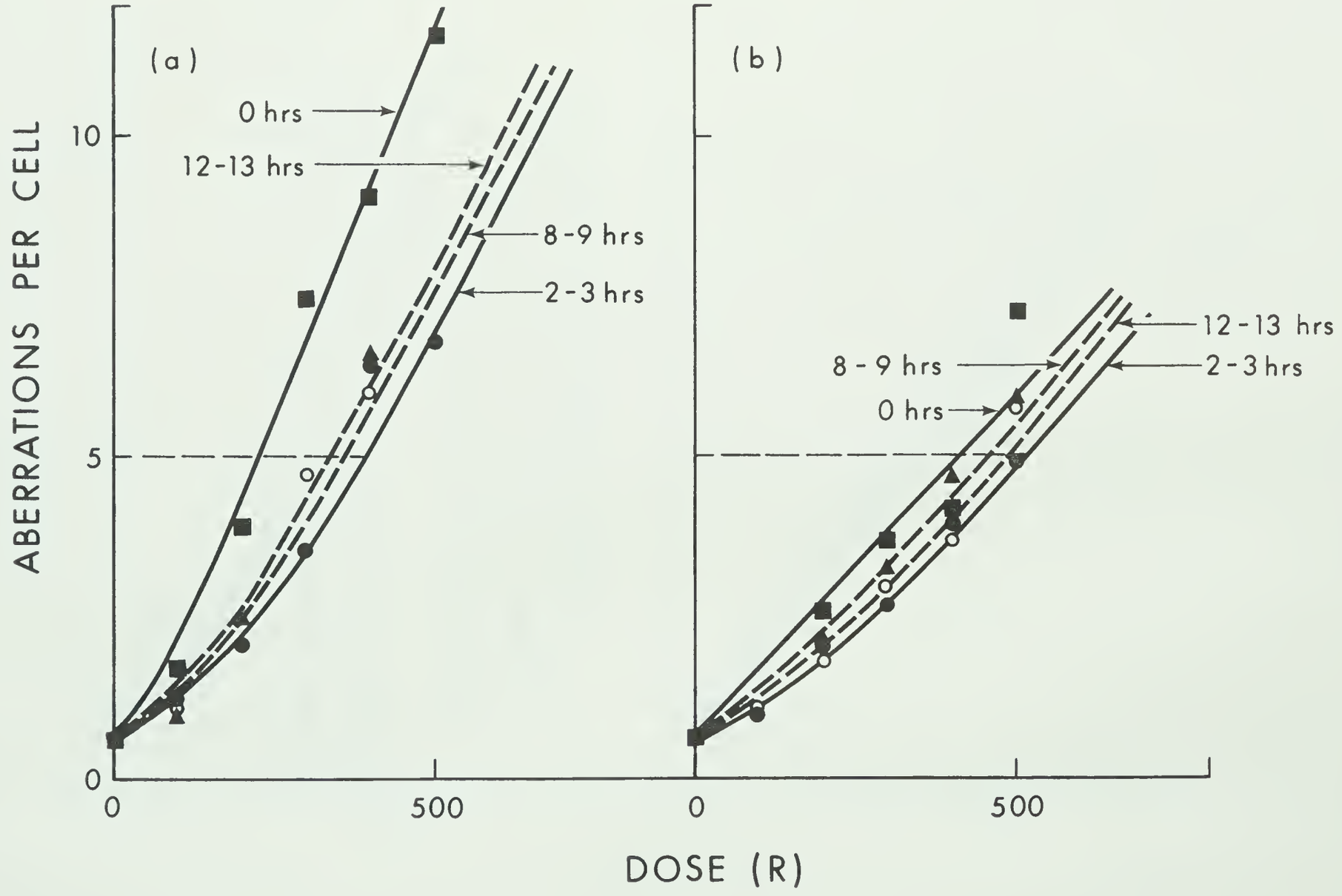

(a)
(b)
0 hrs
12-13 hrs
8-9 hrs
2-3 hrs
ABERRATIONS PER CELL
10
5
0
0
500
DOSE (R)

Figure 8. Dose-effect curves for chromosomal damage in bifilar 5-BUdR labelled CHO cells irradiated at different times post-synchrony.

a) Cells synchronised in mitosis with colcemid.

b) Cells synchronised in mitosis by shaking.

For symbols refer to legend in Figure 6.

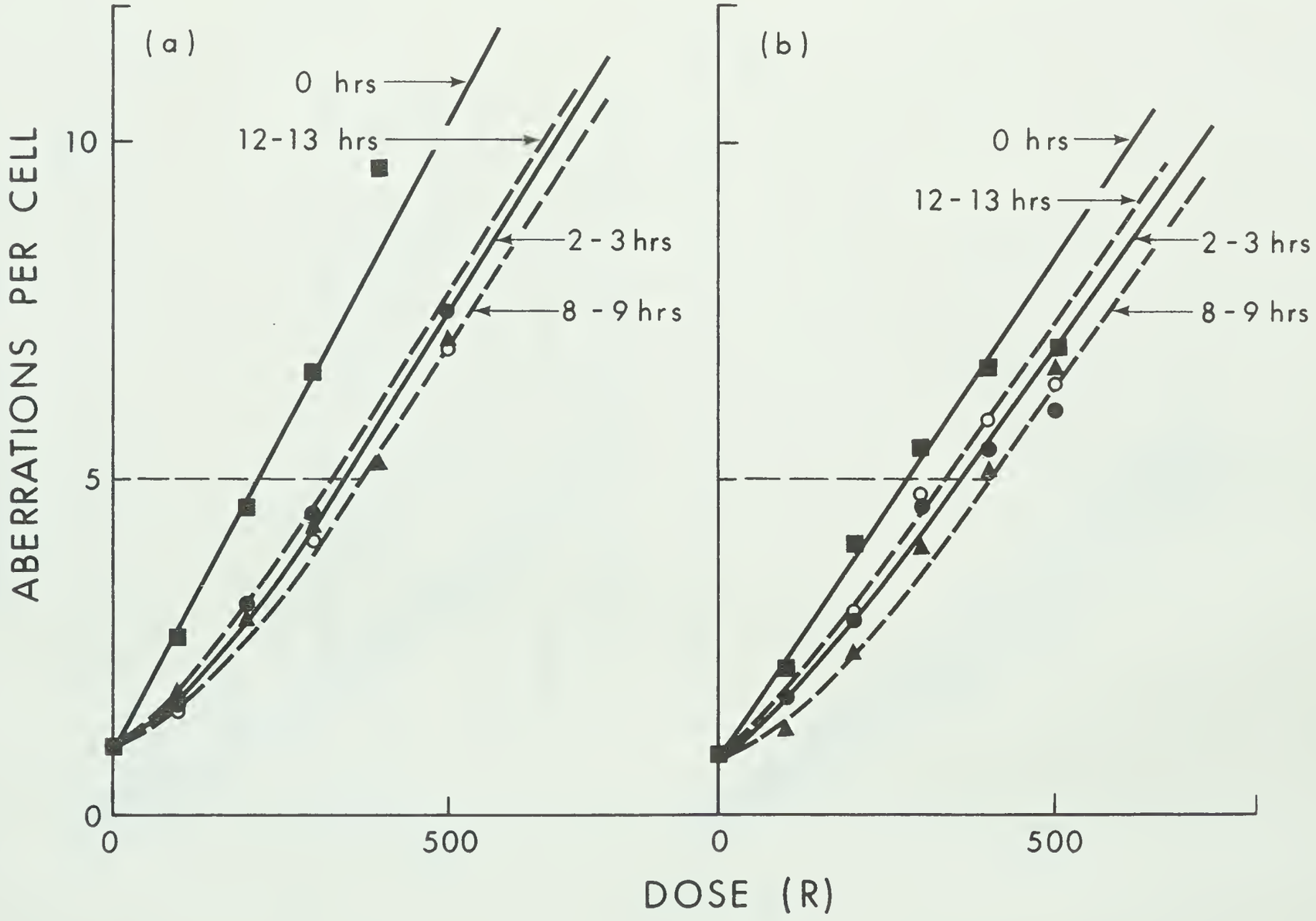

(a)
(b)
0 hrs
12-13 hrs
2-3 hrs
8-9 hrs
ABERRATIONS PER CELL
10
5
0
0
500
DOSE (R)

Figure 9. Dose-effect curves for chromosomal damage in unifilar TdR labelled CHO cells irradiated at different times post-synchrony.

a) Cells synchronised in mitosis with colcemid.

b) Cells synchronised in mitosis by shaking.

For symbols refer to legend in Figure 6.

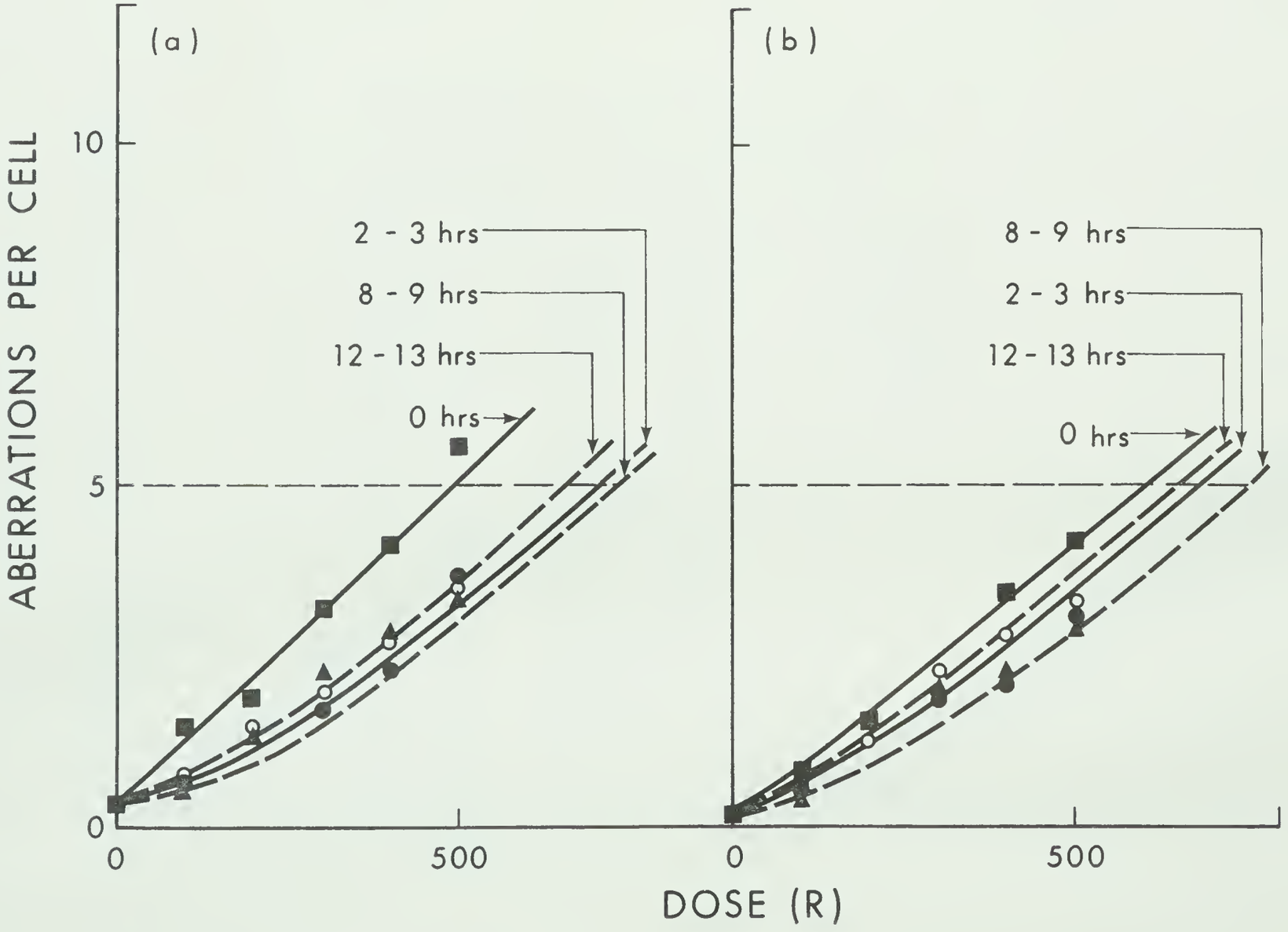

(a)
(b)
ABERRATIONS PER CELL
10
5
0
2 - 3 hrs
8 - 9 hrs
12 - 13 hrs
0 hrs
8 - 9 hrs
2 - 3 hrs
12 - 13 hrs
0 hrs
0
500
0
500
DOSE (R)

Figure 10. Dose-effect curves for chromosomal damage in bifilar TdR labelled CHO cells irradiated at different times post-synchrony.

a) Cells synchronised in mitosis with colcemid.

b) Cells synchronised in mitosis by shaking.

For symbols refer to legend in Figure 6.

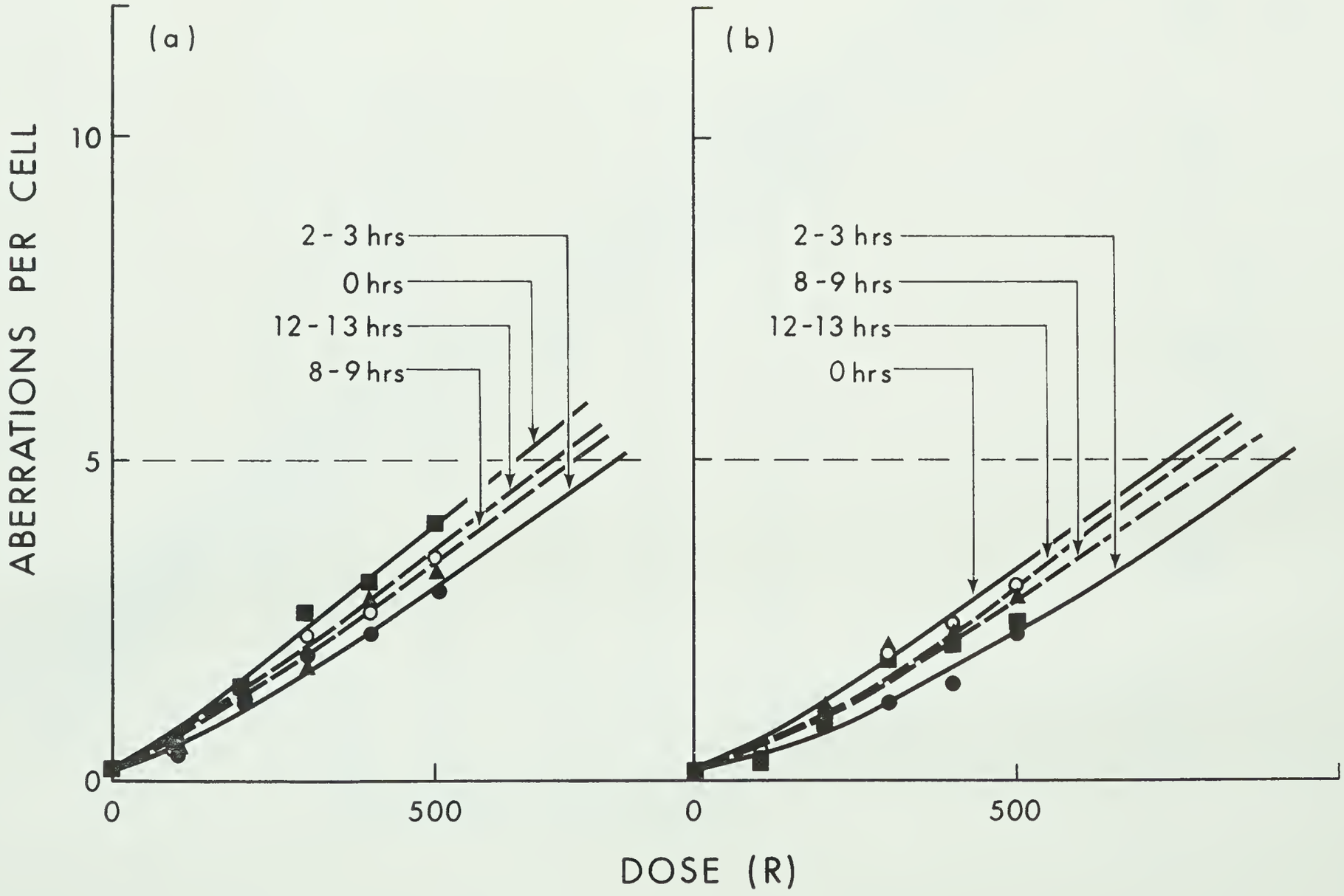

(a)
(b)
ABERRATIONS PER CELL
10
5
0
2 - 3 hrs
0 hrs
12 - 13 hrs
8 - 9 hrs
2 - 3 hrs
8 - 9 hrs
12 - 13 hrs
0 hrs
0
500
0
500
DOSE (R)

Figure 11. Dose-effect curves for survival of normal CHO cells synchronised with colcemid and irradiated at different times post-synchrony.

Squares and solid lines indicate irradiation at mitosis (0 hrs).

Closed circles and dotted lines indicate irradiation at G_1 (2 - 3 hrs).

Open circles and solid lines indicate irradiation at S (8 - 9 hrs).

Triangles and dotted lines indicate irradiation at late S-early G_2 (12 - 13 hrs).

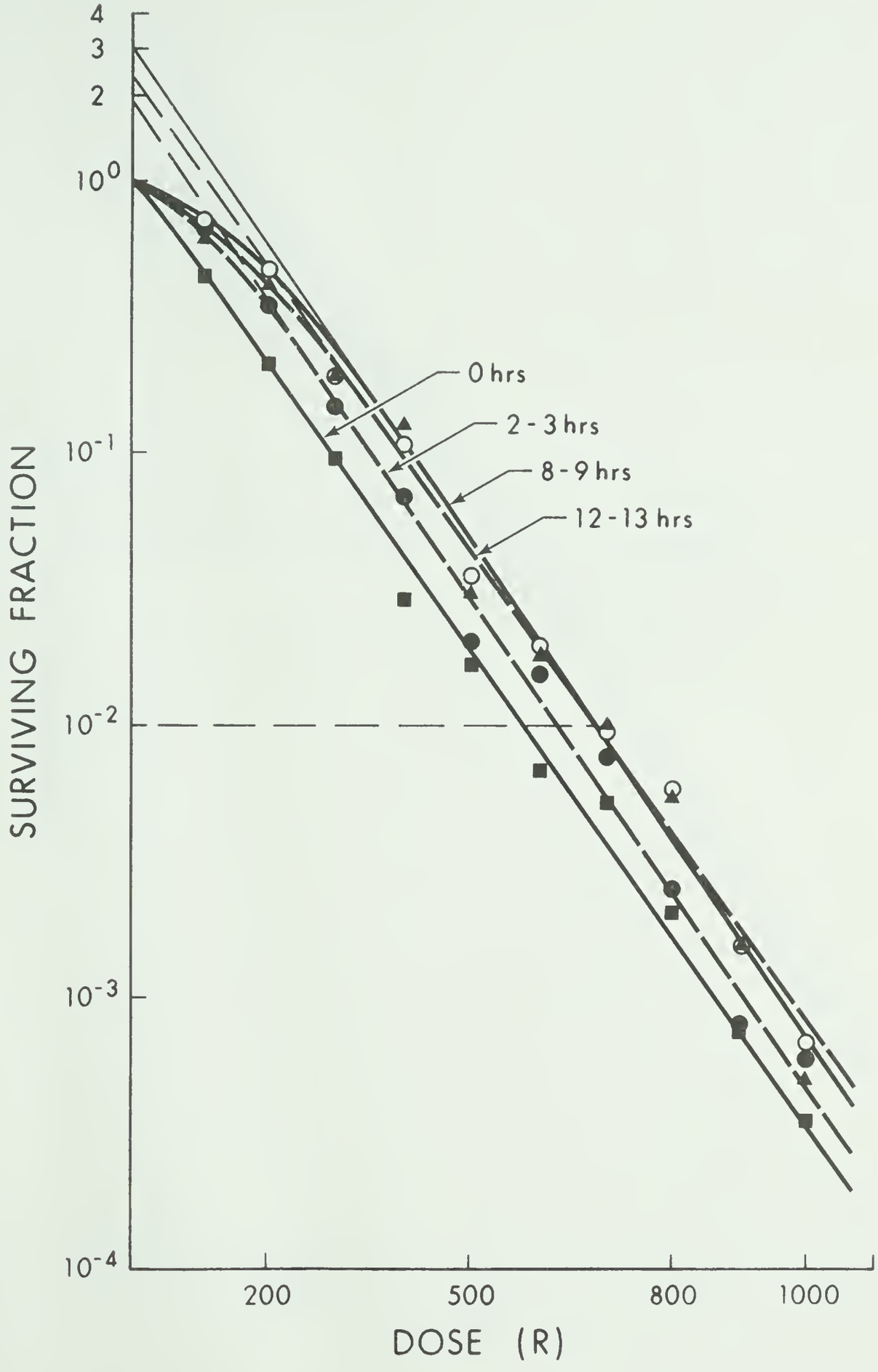

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2 - 3 hrs
8 - 9 hrs
12 - 13 hrs
200
500
800
1000
DOSE (R)

Figure 12. Dose-effect curves for survival of normal CHO cells synchronised by shaking and irradiated at different times post-synchrony.
For symbols refer to legend in Figure 11.

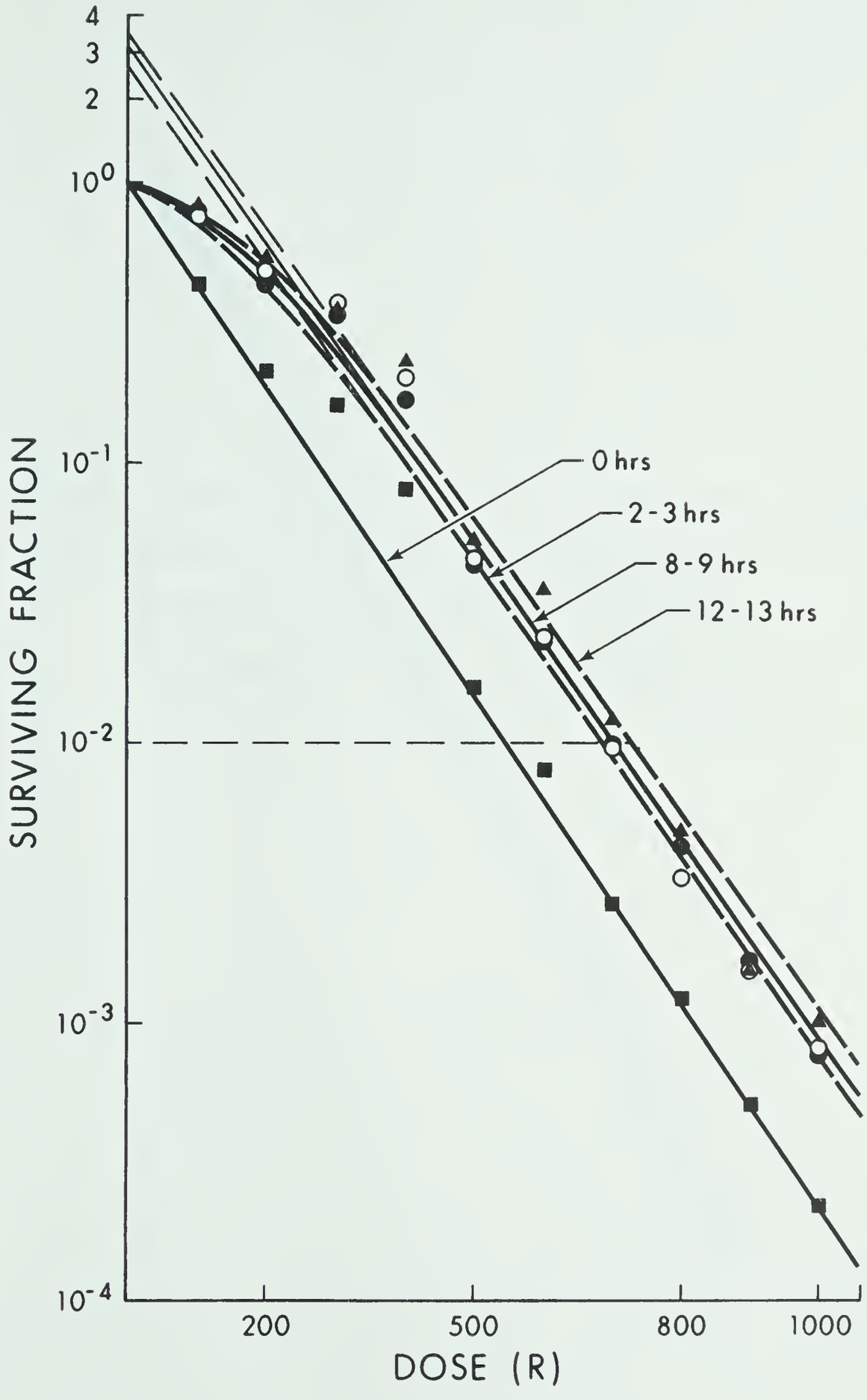

SURVIVING FRACTION
4
3
2
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0 hrs
2-3 hrs
8-9 hrs
12-13 hrs
200
500
800
1000
DOSE (R)

Figure 13. Dose-effect curves for survival of unifilar 5-BUdR labelled CHO cells synchronised with colcemid and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

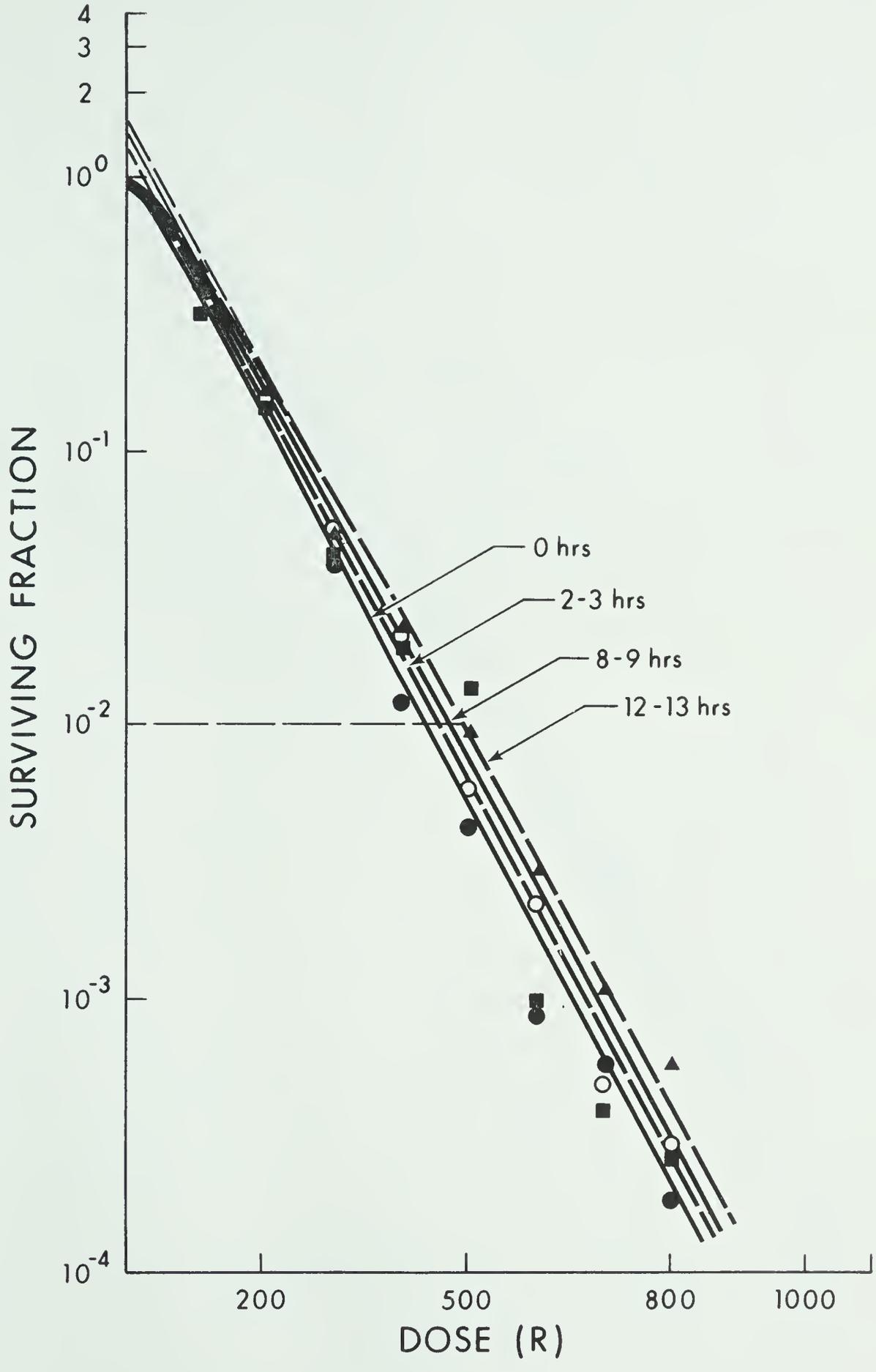

SURVIVING FRACTION
4
3
2
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0 hrs
2-3 hrs
8-9 hrs
12-13 hrs
200
500
800
1000
DOSE (R)

Figure 14. Dose-effect curves for survival of unifilar 5-BUdR labelled CHO cells synchronised by shaking and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

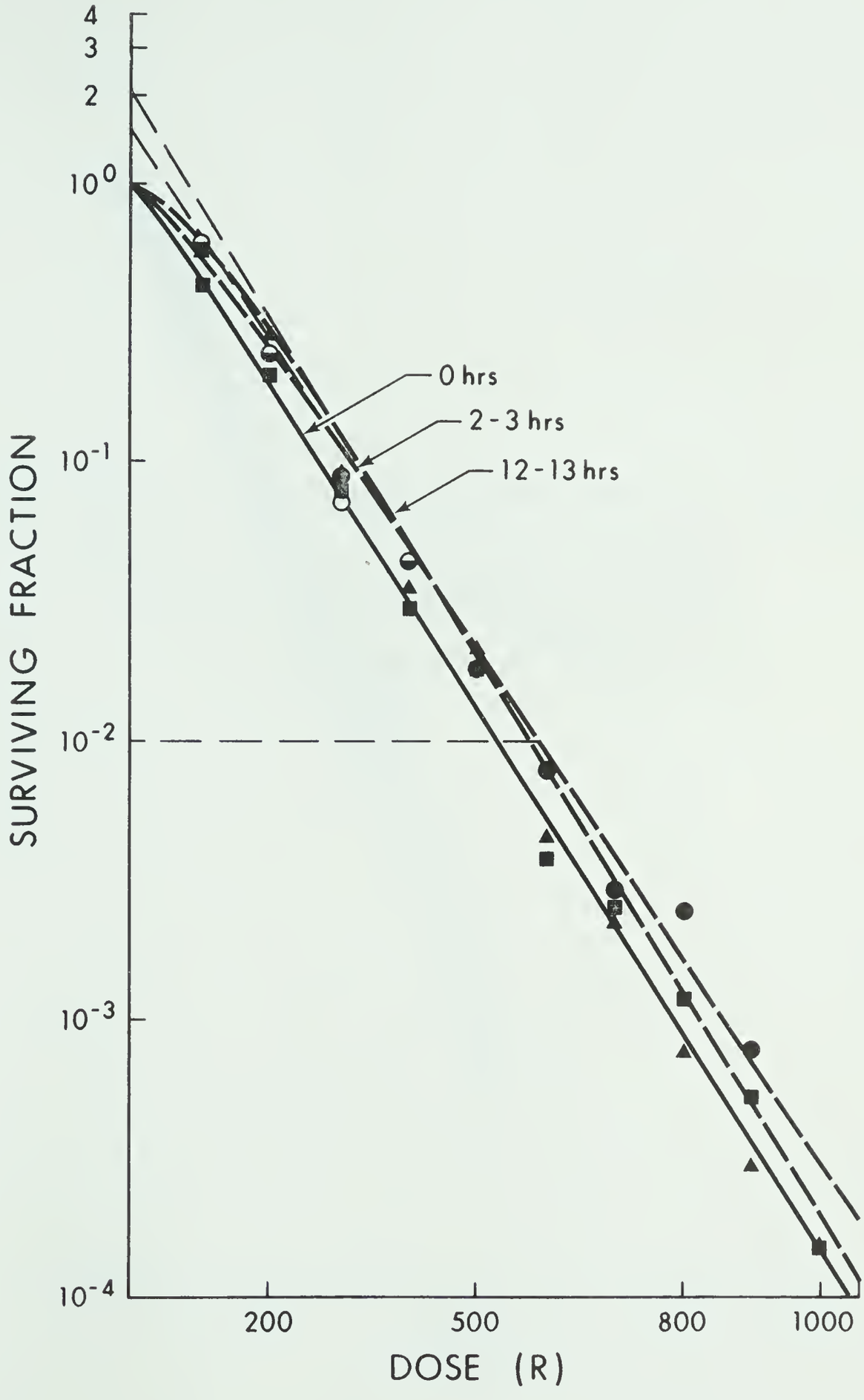

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2 - 3 hrs
12 - 13 hrs
200
500
800
1000
DOSE (R)

Figure 15. Dose-effect curves for survival of bifilar 5-BUdR labelled CHO cells synchronised with colcemid and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

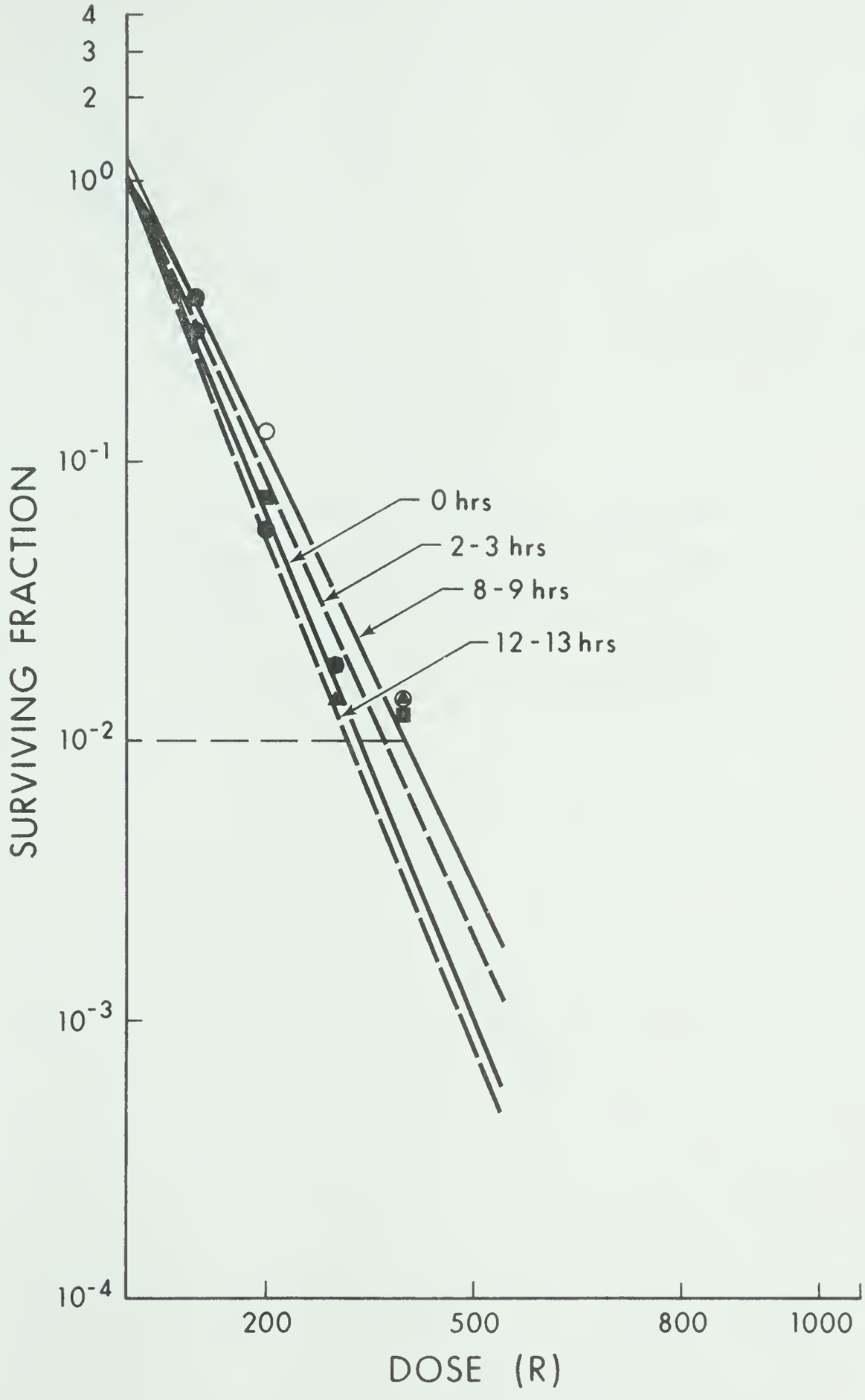

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2-3 hrs
8-9 hrs
12-13 hrs
200
500
800
1000
DOSE (R)

Figure 16. Dose-effect curves for survival of bifilar 5-BUdR labelled CHO cells synchronised by shaking and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

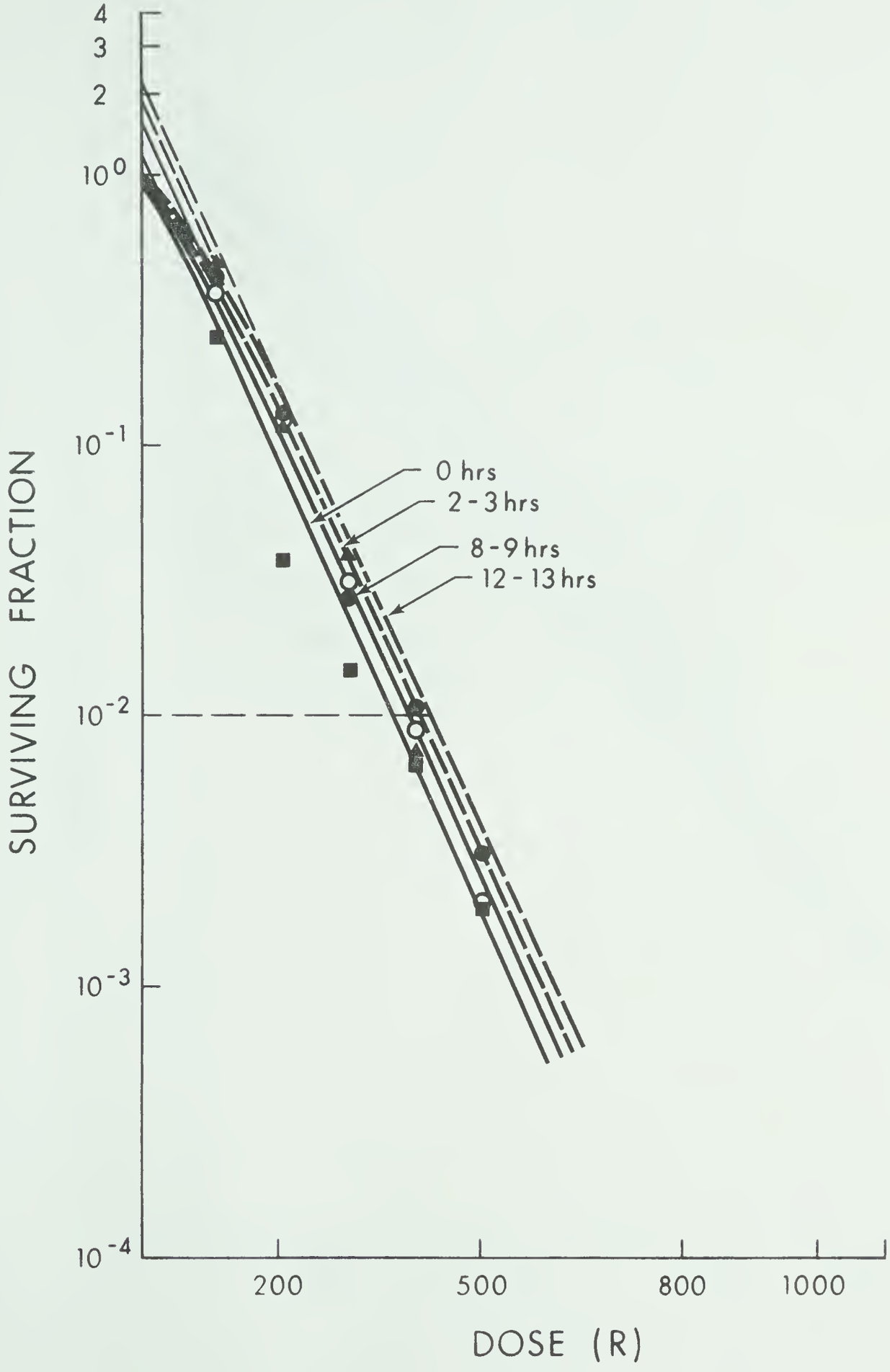

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2 - 3 hrs
8 - 9 hrs
12 - 13 hrs
200
500
800
1000
DOSE (R)

Figure 17. Dose-effect curves for survival of unifilar TdR labelled CHO cells synchronised with colcemid and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

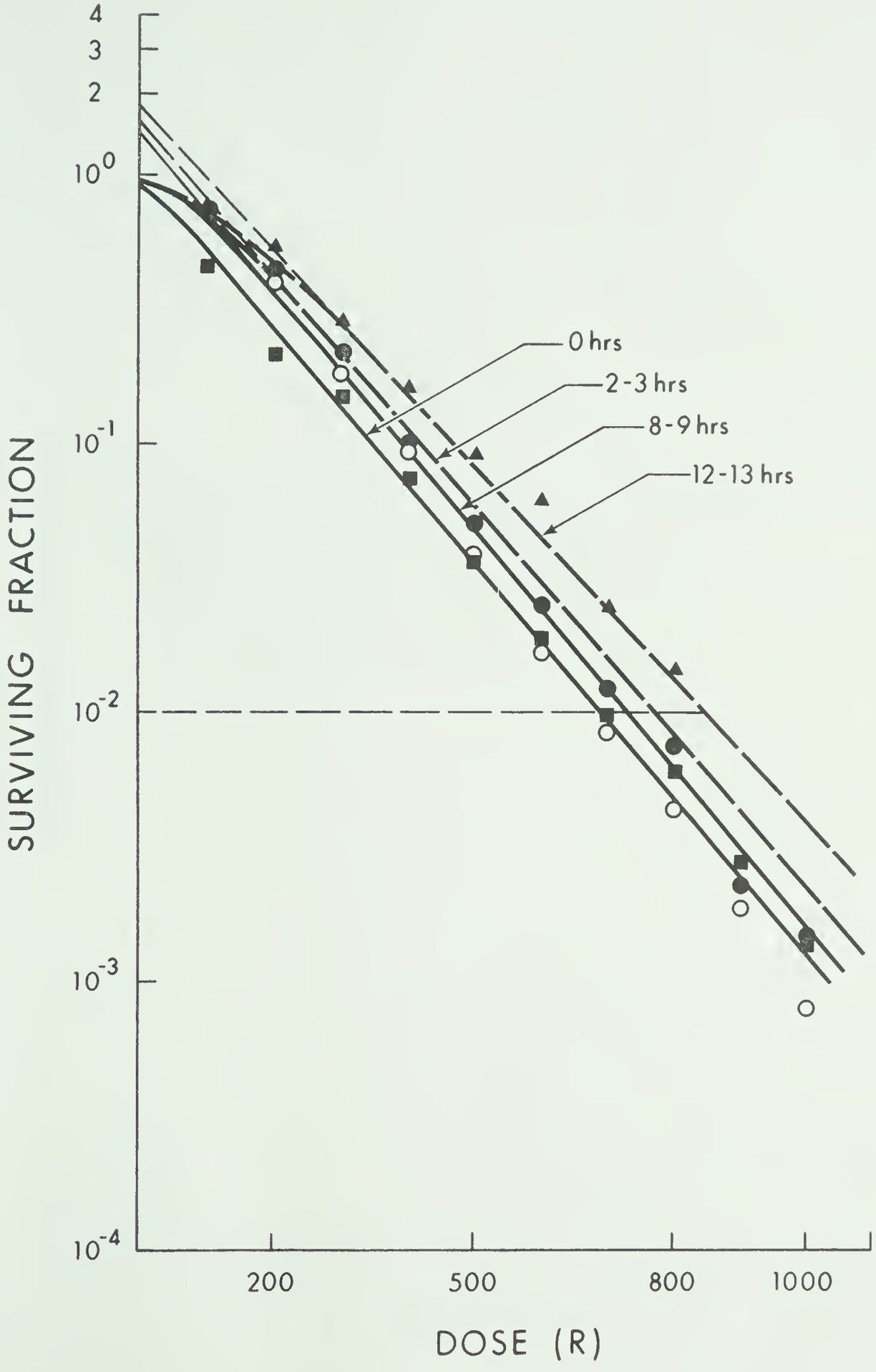

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2-3 hrs
8-9 hrs
12-13 hrs
200
500
800
1000
DOSE (R)

Figure 18. Dose-effect curves for survival of unifilar TdR labelled CHO cells synchronised by shaking and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

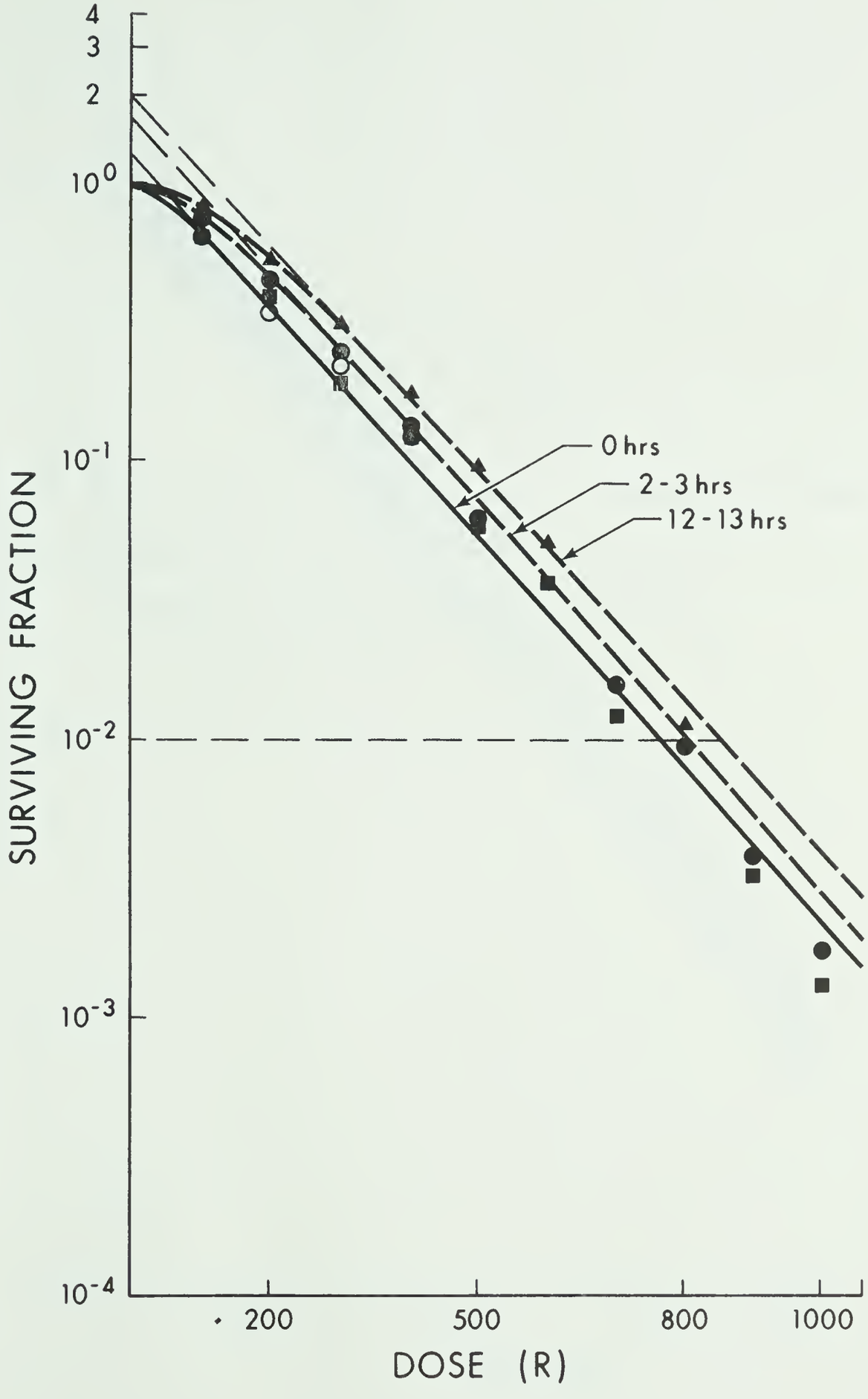

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2 - 3 hrs
12 - 13 hrs
200
500
800
1000
DOSE (R)

Figure 19. Dose-effect curves for survival of bifilar TdR labelled CHO cells synchronised with colcemid and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.

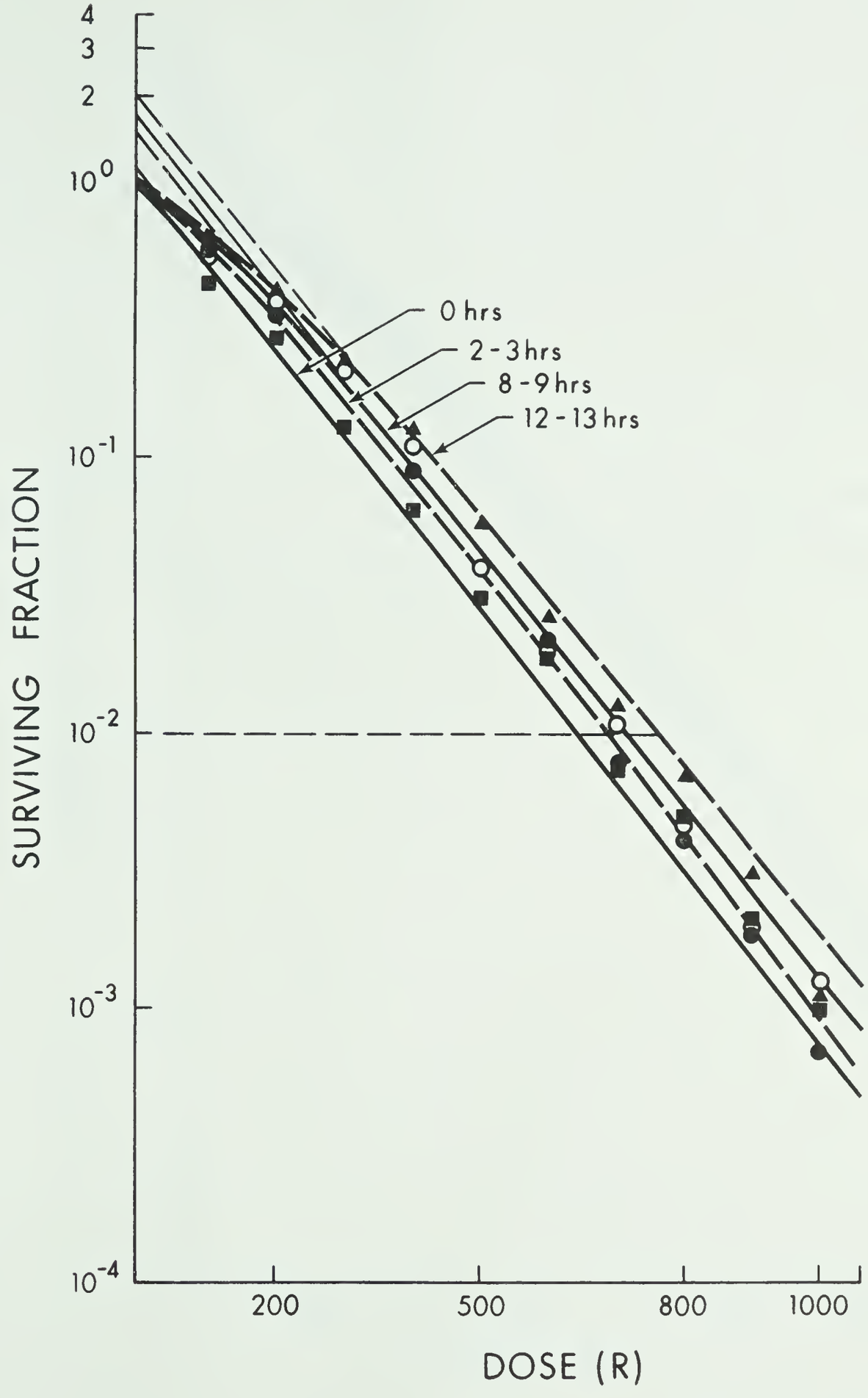
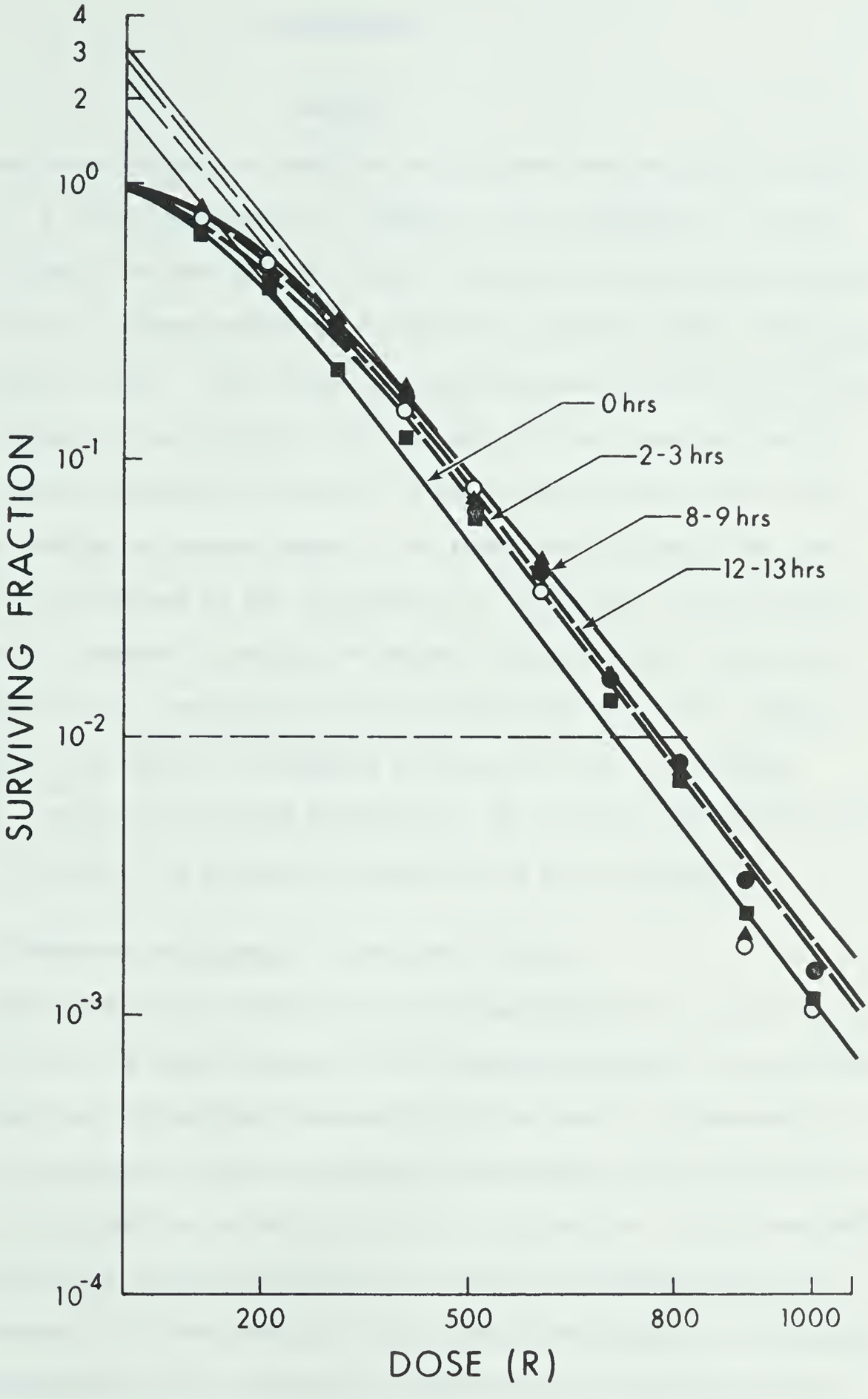

4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2 - 3 hrs
8 - 9 hrs
12 - 13 hrs
200
500
800
1000
DOSE (R)

Figure 20. Dose-effect curves for survival of bifilar TdR labelled CHO cells synchronised by shaking and irradiated at different times post-synchrony. For symbols refer to legend in Figure 11.


4
3
2
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
0 hrs
2-3 hrs
8-9 hrs
12-13 hrs
200
500
800
1000
DOSE (R)

DISCUSSION

General

Many radiation biologists subscribe to the view that radiation induced cell death is a direct consequence of damage to the chromosomes, a theory which has gradually evolved from the early findings that ionising radiations induced structural rearrangements of chromosomes (Koernicke 1905) and point mutations (Muller 1927). Since then, various experiments (Brown and Nelson-Rees 1961, Rogers and von Borstel 1957, and others) have revealed that the nucleus is the most sensitive organelle in proliferating cells and is thus a target for radiation induced damage. The inevitable inference has been that the lesions incurred by the chromosomes in the nucleus are responsible for cell death. However, a paucity of relevant and definitive experimental data, wherein the two consequences of radiosensitivity have been examined simultaneously, has impeded the general acceptance of the "chromosomal aberrations - cellular lethality" hypothesis. The current investigation was undertaken, therefore, to attempt to correlate the two responses.

Chromosomal Damage as a Parameter of Radiosensitivity

The largest number of cellular effects of radiation with visible manifestations by far are those concerned with chromosomal damage. The radiation induced chromosomal aberrations frequently observed are (1) chromosome aberrations which apparently precede chromosome replication, and (2) chromatid aberrations which allegedly occur during and after replication. It is reasonable to expect that their relative proportions, visible at the first division after irradiation, will vary with the time at which the cells are irradiated. Various investigations with synchronised mammalian cell populations have indeed demonstrated that the expression of this response is "age-dependent"

(Dewey *et al.* 1970, 1971, Yu and Sinclair 1967, and others).

The present study reveals that both quantitatively and qualitatively chromosomal damage in cells is dependent upon the time in the cycle at which the cells are irradiated. Qualitatively, the relative proportion of chromosomes to chromatid aberrations is higher in cells irradiated soon after synchronisation but decreases as the synchronised cells traverse the cell cycle. This is not an unexpected occurrence if aberration type is dependent upon whether or not chromosome replication has occurred, since chromosomes are known to replicate asynchronously within the cell cycle (Taylor 1960, and others).

One observation of note with regard to chromosomal aberrations pertains to the use of the mitotic poison colcemid as a synchronising agent. The alkaloid is known to block protein polymerisation and cause disorganisation in the sol-gel structure by binding non-covalently to sites of interaction between the protein sub-units of the spindle (Borisy and Taylor 1967). It has been found also to have an effect on radiation-induced aberration frequencies, which are dependent upon the age of the cell at the time of irradiation (Brumfield 1943 and Davidson 1958). Dewey and Miller (1969) observed a relative increase in chromatid type exchanges in Chinese hamster cells pretreated with colcemid, which they attributed to an uncoiling of the chromosomes as a result of reduced protoplasmic viscosity. This investigation does not reveal an increase in the relative proportion of chromatid exchanges. Instead, a slight overall increase in radiation induced chromosomal damage for the four cyclic phases is indicated when cells are synchronised with colcemid. It is suggested, therefore, that the vulnerability of colcemid treated cells to radiation induced chromosomal damage may be due to a combination of the biochemical and morphological effects of the alkaloid on the

cell and its chromosomes.

A second noteworthy observation concerns the use of the halogenated pyrimidine analogue 5-BUdR. Dewey *et al.* (1971) reported an increase in X-ray induced chromatid exchanges for synchronised 5-BUdR pretreated cells, irradiated at mitosis, which they feel may be related to the same phenomenon responsible for increased chromatid exchanges with colcemid pretreatment. The present study shows that there is a substantial increase in the overall amount of radiation-induced chromosomal damage in cells grown in the presence of the analogue, both for one and for two generations. Furthermore, a substantial increase is evident in the relative proportion of chromatid exchanges for cells irradiated in mitosis and G_1. Cells pretreated with 5-BUdR and synchronised either in the presence or absence of the mitotic poison colcemid both show an increase in the relative proportion of chromatid exchanges. However, since cells synchronised without colcemid do not exhibit a disorganisation in the sol-gel state, it is difficult to envisage the responsible phenomenon as being similar to the one responsible for increased chromatid exchanges in colcemid pretreated cells. Instead, potentiation of the response in cells pretreated with the base analogue may be due to an increase in the inherent susceptibility of DNA to primary damage. Since only a relatively few primary ionisations become registered in DNA sites essential for reproductive integrity it was suggested by Mohler and Elkind (1963) that treatment with 5-BUdR may either sensitise some sites of incorporation which otherwise would not contribute substantially to X-ray sensitivity in the normal cell or the base analogue may impede the repair of damaged sites by interfering with cellular repair processes. They claim support for their hypothesis in the observations that 5-BUdR can produce "hot-spots" of mutation in DNA (Benzer 1961) and it enhances UV killing of

T_1 phage by inhibiting repair processes which otherwise occur after bacterial infection (Howard-Flanders and Boyce 1962). Their suggestion therefore seems feasible, especially since thymine analogues have in themselves been shown to be potent mutagenic agents (Freese 1959, and others). In addition, it has been demonstrated that radiosensitisation occurs in a region of selective incorporation of the base analogue (Dewey *et al.* 1966).

The use of exogenous TdR is alleged by Yang *et al.* (1966) to induce chromosomal aberrations. They claim that the induction of aberrations is directly dependent on the concentration of the exogenous base in the culture medium and suggest that the aberrations may be caused either by a blockage in DNA synthesis or by an imbalance in the nucleotide pool. Experiments in the current investigation, where cells were grown in exogenous TdR (3.4 µg/ml) for one and two generations, do not indicate an enhancement of radiation induced chromosomal damage. The nature of the response is similar to that of untreated cells, *viz.*, the relative proportion of chromosome aberrations and chromatid aberrations is dependent upon the cyclic position of the cell at irradiation. The relative proportions of the two types of aberrations in the four cyclic phases are not significantly different from their proportions in the respective phases of untreated cells. Neither are the frequencies of the aberrations affected by the method of synchrony nor are they altered by the duration of growth of the cells in the exogenous base.

Cells irradiated in mitosis were observed to be most sensitive in the expression of this response. The irradiation of cells as they traversed the cycle showed reduced expression of response. The expression of the response for pretreated 5-BUdR cells was enhanced in all cyclic phases. Cells grown in exogenous TdR expressed a response similar to that of "normal" (untreated) cells.

Cell Survival as another Parameter of Radiosensitivity

Another biological manifestation of cellular radiation damage is the loss of proliferative ability. Only a fraction of a statistically significant (large enough) population of irradiated cells continue to divide and give rise to colonies. An assay of these colonies serves as a criterion for the assessment of loss of reproductive integrity. The method for clonal growth of X-irradiated mammalian cells *in vitro* permits such as assessment (Puck *et al.* 1956).

Data obtained from an assay of viable X-irradiated cells by colony formation are utilised in the preparation of dose-effect curves. These survival curves, plotted as the log of survival *vs.* linear dose are usually sigmoidal, characterised by a "quasi-threshold" or "shoulder" in the region of low doses followed by a region of exponential response (Elkind and Whitmore 1967).

In addition to causing chromosomal aberrations that may be responsible for cell death, X-irradiation induces sub-lethal lesions that do not result in aberrations or cellular lethality unless they interact with lesions produced by subsequent doses (Elkind and Sutton 1960). The shoulder on survival curves is indicative of an accumulation of a certain amount of sub-lethal damage before reproductive integrity is affected (Elkind and Whitmore 1967). Thus surviving cells must be cells that, although affected, have not accumulated a sufficient amount of sub-lethal damage to cause the loss of reproductive integrity. Or they may be cells that possess the capacity to repair the sub-lethal damage before first division irradiation. Evidence for the repair of sub-lethal damage has been demonstrated by the extensive dose-fractionation studies of Elkind and associates (Elkind and Sutton 1960, Elkind *et al.* 1964, and others).

The dose-effect curves for survival response obtained in this study are of the threshold type. In addition, the response is found to be age-dependent, as evidenced by the nature of the response for cells irradiated at various times in the cycle. The curves differ with respect to 'shoulder width', extrapolation or 'hitness' number and the slope of the exponential region.

A prominent feature of the dose-effect curve for cells irradiated in mitosis is the absence of the 'quasi threshold' or 'shoulder' region. If a shoulder is indicative of reduced killing at low doses because of the necessity for accumulation of sub-lethal damage, its absence should imply increased killing at low doses. This may be due to a reduced capacity in mitotic cells for repair of radiation-induced damage. As a corollary, dose-effect curves with different 'shoulder-widths' should imply varying degrees of capacity for repair of damage. The current study shows that the curves for cells irradiated at other times in the cycle have varying 'shoulder-widths'. Some investigations have shown that cells irradiated during the S phase have the largest shoulder width on the survival curve. The capacity for repair during this phase was also found to be the greatest (Dewey *et al.* 1970, 1971, Sinclair and Morton 1966, and others). The inference that the degree of capacity for repair of radiation induced sub-lethal damage varies with 'shoulder-width' therefore seems feasible. The speculation that repair processes may be associated with the DNA synthetic process (Belli and Shelton 1969, and others), thus may also be reasonable.

The 'shoulder width' is usually specified by the dose at which the backward extrapolation intersects the abscissa and a measure of this is indicative of capacity for recovery from sub-lethal damage. Modification of radiation response is indicated by a reduction in this 'shoulder width'

which in turn implies an enhancement of radiation-induced damage. Radiobiological studies with base analogues represent a specific change in nucleic acid structure associated with a modification in radiosensitivity. Investigations with Chinese hamster cells pretreated with 5-BUdR for one generation have revealed that the cyclic response is altered in such a way that there is a reduction in the width of the shoulder (Dewey *et al.* 1971). The current study, wherein cells were pretreated with the base analogue for one and two generations, also reveals a reduction in 'shoulder width' for survival response with a concomitant enhancement of the lethal response in cells of all ages. The increased radiosensitisation could be attributed to an interference by the base analogue with cellular repair mechanisms (Mohler and Elkind 1963, and others). As an alternative, the reduction in survival caused by pretreatment with 5-BUdR may be due to a killing or growth inhibiting property of the base analogue. In so doing the system may be selecting for cells more responsive to X-rays, capable of growing in the presence of the analogue. However, the several investigations have not unequivocally established that radiation response is enhanced simply because the base analogues are incorporated into DNA.

Modification in 'shoulder width' is also indicated by a variation in extrapolation or 'hitness' numbers (n). These values are determined by extrapolating the exponential region of the curve back to zero dose to intersect the ordinate axis (Alper *et al.* 1960). These values represent the minimum number of hits necessary to kill a cell. The investigation reveals that the variation in 'shoulder width' is characterised by a variation in 'hitness' numbers for the different cyclic phases.

The cyclic responses for cells grown in exogenous TdR do not reveal a modification in radiation-induced cellular lethality. Cells irradiated

in mitosis still demonstrate the greatest degree of damage and the dose-effect curve lacks the 'quasi threshold' region. The cyclic responses for cells irradiated at other points in the cycle exhibit shoulders of definite width. That exogenous TdR is incorporated into DNA is evident from the analogue replacement experiments. Therefore, since neither of the two responses, cellular lethality and chromosomal damage, are modified by incorporation of exogenous base, it is concluded that TdR is not a radiosensitising compound.

The Two Parameters Correlated

The investigation of each response in itself affords a limited insight into the biological mechanisms of action of ionising radiations. If the two responses can be shown to be directly correlated, then the general acceptance of the "chromosomal aberrations - cellular lethality" hypothesis may be facilitated. However, to effectively attempt to correlate proliferative death with chromosomal damage, not only is a knowledge of how aberrations are manifest post-irradiation necessary, but the nature of aberrations that persist in irradiated cells for several generations is also of obvious importance.

Although the mechanism(s) by which chromosome aberrations are produced is still unknown, it has been observed that spontaneous aberrations in mammalian cells grown *in vitro* are characterised by high frequencies of the chromatid variety. The lack of chromosome aberrations would tend to suggest that an efficient process is in operation which either removes cells with such abnormalities or prevents them from subsequent division (Bender and Gooch 1962, 1963, Elkind and Whitmore 1967, Nowell and Cole 1963). This investigation demonstrates that both cell survival and chromosomal damage

are "age-dependent." For each response, it is indicated that the mitotic phase of the cycle is most radiosensitive. The prevalent type of aberration detectable at the first division post-irradiation, in irradiated mitotic cells, is of the chromosome variety. Radioresistance is suggested to increase with traverse through the cycle till DNA synthesis is terminated. Maximum survival, as suggested by an assay of viable colonies, is found to occur at some point during the S phase. The predominant aberration observable at first metaphase after irradiation of cells in S is of the chromatid type. If there is merit to the suggestion that chromosome aberrations are selected against, then it may seem plausible to speculate that increased radiosensitivity of mitotic cells, indicated by a reduction in survival, may be due to the lethal nature of such aberrations. Conversely, reduced sensitivity of cells in S may be due to the less lethal effects of chromatid aberrations which therefore are the most likely to persist. Experiments with 5-BUdR reveal a relative increase in the proportion of chromatid aberrations for cells irradiated in mitosis, along with an overall increase in chromosomal damage. Survival response is also affected as a result of pretreatment with the base analogue in that there is an enhanced reduction in survival. These observations would seem to weaken the contention that chromatid type aberrations are less lethal as far as cell survival is concerned. However, to demonstrate the quality of aberration that persists in cells after irradiation it would be necessary to examine the cells of surviving colonies for chromosomal aberrations. Further, it may prove informative to examine synchronised irradiated cultures over subsequent generations for chromosomal abnormalities. Such information should provide some insight into the perplexing question of whether the two radiation responses are directly correlated.

CONCLUSION

This investigation demonstrates that the frequency and relative proportion of the two types of aberrations seen in irradiated cell populations vary with the age of the cell at the time of irradiation as do the ability to proliferate and give rise to viable colonies. The expression of the two responses in normal cells is not altered drastically by the use of colcemid as a synchronising agent, although a slight increase in the overall amount of chromosomal damage is observed. The nature of the responses in cells pretreated with 5-BUdR is significantly different from that of normal cells, while that of cells pretreated with TdR is not. It is therefore concluded that 5-BUdR is a radiosensitising compound while TdR is not.

The study lends added support to the claim that the nucleus in cells is a target for ionising radiations. Although it has not been established without ambiguity that DNA is the principal target, this still does not preclude a significant role for the chromosomes in cell killing. Whether or not lethal damage is manifest directly in chromosomes, if biological lesions ultimately give rise to severely damaged chromosomes it is possible that the capacity for unlimited proliferative activity will be lost.

The comprehensive conclusion sought with regard to the degree of correlation between specificity of radiation-induced lesions within the chromosomes and loss of reproductive integrity has not been established. Therefore, it is suggested that caution be exercised in implicating chromosomal damage as the primary cause of cellular lethality. The answers to the puzzling problems of (1) the molecular organisation of mammalian chromosomes, (2) the mechanisms by which abnormalities are produced within these chromosomes, and (3) the capacity and the mechanisms for repair of

radiation-induced damage may provide the necessary evidence for the general acceptance of the "chromosomal aberrations - cellular lethality" hypothesis. The possibility of cell death as a consequence of genetic point mutations and/or damage to other cytoplasmic organelles, however, should not be overlooked.

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APPENDIX

Tables i - x show the raw data for chromosomal aberrations as detected in the various systems.

Tables xi - xv show the raw data for survival response in the various systems.

Table xvi shows a sample calculation for analogue replacement.

Tables xvii - xxi show the data for uptake of ^{3}H TdR by synchronised cells of the various systems.

Table i

TYPE OF TREATMENT - Normal Colcemid

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	57	5	3	-	3	-	-	66	14	0.21	Mitosis (0 hrs)
100	18	9	34	-	14	-	-	43	71	1.44	
200	10	2	60	1	19	0	-	33	102	2.88	
300	8	7	39	8	23	3	1	35	122	3.27	
400	9	5	74	3	20	2	1	35	135	3.64	
500	3	11	60	6	33	5	3	30	175	5.62	
100	24	8	13	6	6	-	2	47	49	0.83	G_1 phase (2-3 hrs)
200	13	2	35	4	19	1	1	44	89	1.81	
300	11	10	30	10	26	4	2	45	132	2.72	
400	4	13	30	4	25	4	4	35	125	3.36	
500	1	9	42	15	36	2	3	32	167	5.00	
100	31	13	7	6	3	-	-	46	38	0.61	S phase (8-9 hrs)
200	19	17	8	4	8	-	-	42	49	0.80	
300	23	22	9	41	8	-	-	61	129	1.90	
400	11	10	12	25	3	-	1	36	80	2.01	
500	6	14	14	74	3	-	-	43	182	4.01	
100	25	11	6	11	5	-	-	41	49	0.98	late S—early G_2 (12-13 hrs)
200	24	9	6	27	1	-	-	50	71	1.21	
300	16	17	14	38	2	-	-	43	111	2.37	
400	8	6	7	42	3	-	-	31	103	3.11	
500	+	+	+	+	+	+	+	+	+	+	

- none observed
+ poor sample

Table ii

TYPE OF TREATMENT - Normal Shaking

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	36	7	3	-	1	-	-	45	12	0.27	Mitosis (0 hrs)
100	33	6	18	-	23	-	1	67	72	0.80	
200	16	5	40	-	19	1	3	44	93	1.84	
300	11	5	32	1	25	1	1	35	100	2.59	
400	9	7	57	4	30	3	4	41	152	3.43	
500	4	6	70	2	30	3	4	34	160	4.43	
100	21	4	21	-	8	1	1	41	47	0.87	G_1 phase (2-3 hrs)
200	16	7	23	-	7	-	3	37	50	1.08	
300	7	1	38	3	14	-	1	34	75	1.91	
400	11	6	46	11	22	-	3	39	124	2.91	
500	3	8	72	5	30	-	10	44	170	3.59	
100	18	7	19	4	2	-	-	40	38	0.68	S phase (8-9 hrs)
200	15	5	12	14	3	-	2	37	55	1.21	
300	4	10	20	15	6	-	2	35	76	1.90	
400	4	12	32	22	5	-	2	35	102	2.64	
500	4	14	36	57	11	-	3	47	192	3.82	
100	22	8	16	4	4	-	-	45	40	0.61	late S—early G_2 (12-13 hrs)
200	17	5	18	7	6	-	1	40	51	1.00	
300	12	6	21	22	13	-	1	49	100	1.77	
400	7	10	25	24	1	-	1	31	87	2.53	
500	5	12	26	60	13	-	3	50	190	3.67	

- none observed

Table iii

TYPE OF TREATMENT - Unifilar 5-BUdR Colcemid

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	43	2	11	4	10	-	-	65	41	0.63	Mitosis (0 hrs)
100	17	4	38	10	24	-	2	48	114	1.74	
200	5	3	42	12	48	8	4	45	205	3.93	
300	1	2	93	20	57	8	7	36	293	7.50	
400	+	+	+	+	+	+	+	+	+	+	
500	-	3	73	15	46	7	9	20	244	11.57	
100	14	4	25	9	20	-	3	50	93	1.23	G_1 phase (2-3 hrs)
200	9	8	28	6	30	1	3	43	118	2.11	
300	3	3	24	23	37	2	3	38	161	3.60	
400	-	11	52	45	54	3	5	40	283	6.44	
500	-	2	63	35	60	2	6	37	275	6.83	
100	27	2	25	16	17	-	-	58	93	0.97	S phase (8-9 hrs)
200	9	5	41	25	17	-	6	46	142	2.46	
300	6	9	32	25	25	2	6	38	161	3.60	
400	1	8	47	46	44	3	4	35	255	6.65	
500	-	12	50	35	50	1	5	36	246	6.60	
100	17	2	22	14	11	-	2	46	81	1.13	late S—early G_2 (12-13 hrs)
200	12	7	30	33	16	1	4	46	147	2.56	
300	2	12	53	45	30	-	3	41	221	4.76	
400	-	5	61	51	26	-	7	35	234	6.05	
500	-	6	30	40	32	2	6	31	200	5.82	

- none observed

+ poor sample

Table iv

TYPE OF TREATMENT - Unifilar 5-BUdR Shaking

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	35	4	14	1	8	-	-	57	36	0.63	Mitosis (0 hrs)
100	24	2	30	2	20	-	1	47	78	1.03	
200	11	3	50	6	40	3	4	51	165	2.60	
300	8	2	60	8	64	3	6	53	230	3.71	
400	5	2	76	13	50	2	3	45	218	4.21	
500	-	2	56	10	59	10	4	31	244	7.27	
100	22	3	19	4	29	-	3	56	94	1.05	G_1 phase (2-3 hrs)
200	10	4	30	7	38	22	3	51	138	2.07	
300	6	3	50	9	37	2	3	47	159	2.75	
400	+	+	+	+	+	+	+	+	+	+	
500	1	4	67	17	42	5	6	40	221	4.99	
100	21	4	17	19	10	-	3	50	85	1.07	S phase (8-9 hrs)
200	+	+	+	+	+	+	+	+	+	+	
300	+	+	+	+	+	+	+	+	+	+	
400	2	6	52	42	44	5	8	50	266	4.69	
500	3	11	55	58	55	6	4	50	325	5.87	
100	16	2	19	14	15	-	2	47	83	1.13	late S—early G_2 (12-13 hrs)
200	13	2	30	24	21	-	2	50	126	1.89	
300	6	2	36	40	17	1	2	44	160	3.00	
400	2	6	40	37	26	2	3	43	186	3.69	
500	-	9	40	53	35	4	9	40	259	5.84	

- none observed

+ poor sample

Table v

TYPE OF TREATMENT - Bifilar 5-BUdR Colcemid

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	23	2	6	4	12	-	-	40	40	1.00	Mitosis (0 hrs)
100	7	3	25	18	33	1	6	40	146	2.65	
200	2	2	32	26	46	3	3	35	196	4.60	
300	2	5	55	25	40	2	6	30	230	6.66	
400	-	6	60	45	60	8	5	30	318	9.60	
500	-	8	65	40	50	10	6	30	305	9.10	
100	7	2	12	15	22	1	2	35	96	1.74	G_1 phase (2-3 hrs)
200	2	2	17	27	35	3	2	33	139	3.21	
300	-	4	40	18	33	3	5	31	168	4.42	
400	-	5	40	25	36	3	7	35	193	4.51	
500	+	+	+	+	+	+	+	+	+	+	
100	5	4	10	15	19	-	1	30	84	1.80	S phase (8-9 hrs)
200	2	5	30	32	16	-	5	35	141	3.02	
300	-	-	30	40	24	2	6	33	178	4.39	
400	-	5	35	45	25	-	4	30	188	5.26	
500	+	+	+	+	+	+	+	+	+	+	
100	6	2	12	16	15	-	1	30	78	1.60	late S—early G_2 (12-13 hrs)
200	2	4	20	30	16	1	3	30	126	3.20	
300	+	+	+	+	+	+	+	+	+	+	
400	-	8	25	65	20	1	2	30	215	6.10	
500	-	6	20	65	35	1	4	30	242	7.06	

- none observed
+ poor sample

Table vi

TYPE OF TREATMENT - Bifilar 5-BUdR Shaking

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	30	3	5	5	14	-	2	52	50	0.96	Mitosis (0 hrs)
100	14	-	25	9	44	3	9	50	161	2.26	
200	5	7	44	18	52	3	8	43	219	4.13	
300	2	4	50	14	41	6	6	31	200	5.49	
400	--	4	48	26	57	8	10	35	270	6.75	
500	--	3	65	19	54	9	5	32	250	6.95	
100	10	4	19	13	42	-	3	50	139	1.82	G_1 phase (2-3 hrs)
200	3	5	41	9	49	3	10	50	194	2.92	
300	2	8	40	29	67	5	10	50	280	4.64	
400	-	3	51	19	64	6	6	40	256	5.44	
500	-	2	45	28	63	10	6	40	281	6.06	
100	14	4	18	16	17	1	9	48	110	1.33	S phase (8-9 hrs)
200	4	2	28	26	21	1	6	40	140	2.54	
300	+	+	+	+	+	+	+	+	+	+	
400	-	3	30	42	42	1	5	35	215	5.18	
500	-	6	45	57	28	1	6	30	231	6.74	
100	13	4	14	24	30	-	6	50	138	1.80	late S—early G_2 (12-13 hrs)
200	7	6	26	43	29	3	6	50	200	3.04	
300	3	10	40	55	30	-	5	40	230	4.79	
400	1	5	34	74	24	-	13	38	261	5.90	
500	-	7	33	71	36	2	6	37	274	6.44	

- none observed
+ poor sample

Table vii

TYPE OF TREATMENT - Bifilar TdR Shaking

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	51	4	3	2	1	-	-	60	12	0.20	Mitosis (0 hrs)
100	31	6	10	-	8	-	1	50	28	0.36	
200	23	1	13	2	12	-	6	47	54	0.95	
300	14	4	24	5	27	2	3	50	106	1.92	
400	10	3	33	2	25	-	5	42	100	2.18	
500	10	4	35	5	28	2	6	45	125	2.47	
100	31	4	7	-	8	-	2	50	31	0.42	G_1 phase (2-3 hrs)
200	25	2	18	2	17	-	3	50	64	1.08	
300	22	5	30	-	15	-	3	50	71	1.22	
400	13	3	26	3	18	-	2	43	75	1.54	
500	10	4	26	6	21	-	2	44	110	2.30	
100	32	3	18	2	3	-	-	50	31	0.42	S phase (8-9 hrs)
200	20	2	15	12	11	-	4	50	71	1.22	
300	16	3	38	27	9	-	3	50	119	2.18	
400	12	4	25	23	10	-	2	40	99	2.27	
500	8	2	30	25	16	-	6	40	126	2.95	
100	30	2	19	4	3	-	1	50	37	0.54	late S—early G_2 (12-13 hrs)
200	21	2	24	12	5	-	3	45	66	1.26	
300	15	8	22	29	7	-	4	49	110	2.04	
400	10	4	21	38	7	-	2	46	123	2.47	
500	7	5	21	42	9	-	2	41	136	3.11	

- none observed

Table viii

TYPE OF TREATMENT - Bifilar TdR Colcemid

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	45	2	6	-	1	-	-	50	10	0.20	Mitosis (0 hrs)
100	26	1	6	-	10	-	3	40	33	0.62	
200	19	2	20	6	17	1	4	45	78	1.53	
300	10	-	21	9	29	2	4	40	113	2.62	
400	8	3	30	7	25	2	5	35	115	3.08	
500	5	-	40	4	35	9	4	38	162	4.06	
100	24	1	10	-	6	-	1	38	25	0.46	G_1 phase (2-3 hrs)
200	17	2	12	7	13	-	-	37	54	1.26	
300	9	-	12	10	15	1	4	33	73	2.01	
400	11	2	18	12	22	-	6	39	100	2.36	
500	4	2	22	8	33	1	3	38	120	2.96	
100	26	2	10	4	2	-	1	40	26	0.45	S phase (8-9 hrs)
200	20	3	10	15	10	-	1	40	65	1.42	
300	15	2	12	23	7	-	1	38	76	1.80	
400	8	2	18	36	10	-	2	39	118	2.82	
500	8	2	24	32	12	-	3	35	120	3.22	
100	25	1	15	2	4	-	-	40	28	0.50	late S—early G_2 (12-13 hrs)
200	+	+	+	+	+	+	+	+	+	+	
300	10	2	20	14	20	-	2	38	94	2.27	
400	8	3	25	15	17	1	2	35	100	2.65	
500	5	3	20	33	16	-	4	35	129	3.48	

- none observed
+ poor sample

Table ix

TYPE OF TREATMENT - Unifilar TdR Colcemid

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	31	2	12	-	2	-	-	47	18	0.38	Mitosis (0 hrs)
100	19	5	36	4	20	-	2	50	93	1.48	
200	14	2	32	4	24	-	2	41	94	1.91	
300	+	+	+	+	+	+	+	+	+	+	
400	6	3	57	6	38	-	4	42	192	4.19	
500	6	5	100	4	30	-	3	32	191	5.62	
100	24	8	19	3	8	-	1	50	51	0.64	G_1 phase (2-3 hrs)
200	+	+	+	+	+	+	+	+	+	+	
300	11	6	33	5	19	-	2	44	91	1.78	
400	8	5	45	12	20	1	2	46	122	2.27	
500	7	7	65	15	36	2	-	45	182	3.66	
100	26	5	20	7	5	-	-	56	49	0.49	S phase (8-9 hrs)
200	15	2	22	23	9	-	1	52	90	1.35	
300	12	7	15	33	15	-	1	46	120	2.23	
400	5	7	25	37	20	-	-	45	146	2.86	
500	6	9	40	55	16	-	2	53	196	3.32	
100	24	8	25	10	-	-	-	48	53	0.72	late S—early G_2 (12-13 hrs)
200	10	3	27	13	5	-	-	36	66	1.45	
300	11	10	18	30	6	-	-	42	100	2.00	
400	12	8	32	39	8	-	1	45	136	2.64	
500	8	10	40	52	10	-	4	46	182	3.57	

- none observed

+ poor sample

Table x

TYPE OF TREATMENT - Unifilar TdR Shaking

Dose (R)	Normal cells	Chromatid Gaps	Isolocus deletions	Chromatid Exchanges	CHROMOSOME EXCHANGES			No.of cells	Total No. of breaks	Breaks per cell	Cell Cycle Phase
					Dicentrics	Tricentrics	Rings				
Control (OR)	51	3	9	-	-	-	-	62	12	0.19	Mitosis (0 hrs)
100	27	2	19	4	11	-	-	50	51	0.83	
200	20	3	26	2	24	1	3	51	91	1.59	
300	19	4	30	1	40	3	1	60	130	1.96	
400	8	1	41	2	60	2	6	51	190	3.53	
500	5	4	48	5	43	4	7	40	178	4.26	
100	35	2	12	-	12	-	1	55	40	0.54	G_1 phase (2-3 hrs)
200	21	1	31	-	18	1	1	55	74	1.15	
300	15	5	20	2	25	1	3	41	89	1.98	
400	14	3	30	10	32	-	2	52	121	2.13	
500	7	5	25	6	40	1	7	42	140	3.14	
100	24	3	10	4	2	-	-	38	25	0.47	S phase (8-9 hrs)
200	19	2	15	10	7	-	2	40	55	1.18	
300	12	4	20	22	10	-	2	40	92	2.11	
400	+	+	+	+	+	+	+	+	+	+	
500	7	1	15	22	15	1	1	30	96	3.01	
100	21	5	15	-	8	-	1	40	38	0.76	late S—early G_2 (12-13 hrs)
200	19	2	23	11	15	-	3	55	83	1.31	
300	13	2	40	28	13	-	2	50	128	2.37	
400	6	9	28	28	11	-	4	40	123	2.88	
500	7	5	31	35	21	-	2	43	152	3.40	

- none observed
+ poor sample

Table xi

Dose (R)	Normal Colcemid				Normal Shaking			
	Cell Cycle Phases				Cell Cycle Phases			
	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)
	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction
100	0.455	0.68	0.725	0.61	0.42	0.74	0.73	0.78
200	0.219	0.35	0.47	0.412	0.21	0.43	0.47	0.53
300	0.097	0.15	0.192	0.194	0.16	0.34	0.36	0.35
400	0.031	0.072	0.113	0.132	0.081	0.171	0.199	0.23
500	0.017	0.021	0.036	0.032	0.016	0.045	0.043	0.052
600	0.007	0.016	0.020	0.018	0.008	0.024	0.026	0.035
700	0.0053	0.008	0.0098	0.011	0.0026	0.0095	0.009	0.012
800	0.0021	0.0026	0.006	0.0058	0.0012	0.0043	0.0033	0.0048
900	0.00074	0.0008	0.0016	0.0016	0.0005	0.0017	0.0016	0.0016
1000	0.00036	0.0006	0.0007	0.0005	0.00024	0.00075	0.0008	0.0010
	$\overline{N}$ = 1.38 P.E. = 77% M.I. = 85%				$\overline{N}$ = 1.16 P.E. = 65% M.I. = 75%			

Table xii

Dose (R)	Unifilar 5-BUdR Colcemid				Unifilar 5-BUdR Shaking			
	Cell Cycle Phases				Cell Cycle Phases			
	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)
	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction
100	0.320	0.39	0.465	0.463	0.44	0.60	0.59	0.56
200	0.145	0.140	0.155	0.178	0.21	0.25	0.25	0.29
300	0.04	0.039	0.051	0.0495	0.083	0.09	0.079	0.09
400	0.02	0.013	0.022	0.023	0.030	0.045	0.047	0.036
500	0.014	0.0044	0.006	0.0096	0.018	0.018	—	0.022
600	0.001	0.0009	0.0023	0.0030	0.0038	0.008	—	0.005
700	0.0004	0.0006	0.0005	0.0011	0.0025	0.003	—	0.0024
800	0.00026	0.00014	0.0003	0.0006	0.0012	0.0025	—	0.0008
900	—	—	0.00007	0.0001	0.0005	0.0008	—	0.003
1000	—	—	—	—	0.00015	0.0003	—	0.00015
	$\bar{N}$ = 1.25 P.E. = 45% M.I. = 80%				$\bar{N}$ = 1.30 P.E. = 40% M.I. = 60%			

Table xiii

Dose (R)	Bifilar 5-BUdR Colcemid				Bifilar 5-BUdR Shaking			
	Cell Cycle Phases				Cell Cycle Phases			
	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)
	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction
100	0.37	0.29	0.38	0.29	0.25	0.42	0.38	0.47
200	0.075	0.058	0.13	0.058	0.038	0.135	0.126	0.119
300	0.019	0.019	0.019	0.014	0.015	0.027	0.032	0.04
400	0.013	0.014	0.014	0.014	0.0068	0.011	0.009	0.0075
500	—	—	—	—	0.0020	0.0032	0.0021	0.0032
600	—	—	—	—	—	—	—	—
700	—	—	—	—	—	—	—	—
800	—	—	—	—	—	—	—	—
900	—	—	—	—	—	—	—	—
1000	—	—	—	—	—	—	—	—
	$\bar{N}$ = 1.17 P.E. = 30% M.I. = 79%				$\bar{N}$ = 1.19 P.E. = 35% M.I. = 70%			

Table xiv

Dose (R)	Unifilar TdR Colcemid				Unifilar TdR Shaking			
	Cell Cycle Phases				Cell Cycle Phases			
	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)
	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction
100	0.47	0.73	0.74	0.70	0.66	0.72	0.64	0.79
200	0.220	0.440	0.405	0.548	0.39	0.45	0.34	0.53
300	0.150	0.220	0.185	0.29	0.19	0.25	0.22	0.30
400	0.075	0.100	0.095	0.165	0.13	0.136	0.13	0.176
500	0.038	0.050	0.0395	0.094	0.06	0.063	—	0.096
600	0.019	0.026	0.017	0.064	0.037	0.048	—	0.052
700	0.010	0.0121	0.0086	0.025	0.012	0.016	—	0.016
800	0.0061	0.0077	0.0045	0.015	0.0090	0.0098	—	0.0114
900	0.0029	0.0023	0.0019	—	0.0033	0.0039	—	—
1000	0.0014	0.0015	0.0008	—	0.0013	0.0018	—	0.0018
	$\bar{N}$ = 1.25 P.E. = 45% M.I. = 80%				$\bar{N}$ = 1.32 P.E. = 55% M.I. = 64%			

Table xv

Dose (R)	Bifilar TdR Colcemid				Bifilar TdR Shaking			
	Cell Cycle Phases				Cell Cycle Phases			
	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)	Mitosis (0 hrs)	G_1 phase (2-3 hrs)	S phase (8-9 hrs)	late S—early G_2 (12-13 hrs)
	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction	Survival fraction
100	0.43	0.575	0.56	0.63	0.65	0.79	0.765	0.84
200	0.275	0.33	0.37	0.42	0.44	0.45	0.51	0.567
300	0.13	0.19	0.21	0.229	0.22	0.285	0.311	0.33
400	0.065	0.088	0.115	0.13	0.127	0.170	0.155	0.195
500	0.031	0.039	0.040	0.058	0.070	0.072	0.081	0.076
600	0.022	0.022	0.020	0.027	0.035	0.042	0.035	0.045
700	0.008	0.008	0.011	0.013	0.014	0.017	—	0.0175
800	0.0051	0.0043	0.0048	0.007	0.0075	0.0084	0.0083	0.0091
900	0.0020	0.0019	0.002	0.0031	0.0024	0.0032	0.0019	0.0021
1000	0.0010	0.0007	0.0013	0.0011	0.0012	0.0015	0.0011	0.0018
	$\bar{N}$ = 1.26 P.E. = 45% M.I. = 75%				$\bar{N}$ = 1.24 P.E. = 60% M.I. = 65%			

Table xvi

A sample calculation for analogue replacement

Unifilar TdR labelling

Exogenous cold TdR in medium — 3·4 µg/ml

^{3}H TdR added to medium — 0·5 µCi/ml

No. of cells — 5,300,000

Efficiency of machine — 42%

Screw factor — 100 ÷ 42 = 2·38

Obtained counts per minute (cpm) — 480,000

∴ disintegrations per minute (dpm) — 480,000 x 2·38

dpm/cell — $\frac{480,000 \times 2{\cdot}38}{5,300,000} \equiv 0{\cdot}21$

µmoles per cell is given by the formula:

$$\frac{\text{dpm/cell}}{2{\cdot}2\text{x}10^{6}\ \text{dpm/µCi} \times \text{s.a. in µCi/µg} \times \text{Mol.Wt.}}$$ (Dewey and Humphrey 1965).

∴ µmoles TdR/cell — $\frac{0{\cdot}21\ \text{dpm/cell}}{2{\cdot}2\text{x}10^{6}\ \text{dpm/µCi} \times 0{\cdot}15\ \text{µCi/µg} \times 242}$

— $2{\cdot}7\text{x}10^{-9}$ µmoles TdR/cell

% replacement — $\frac{2{\cdot}7\text{x}10^{-9}}{1{\cdot}4\text{x}10^{-8}} \times 100 = 19\%$

where $1{\cdot}4\text{x}10^{-8}$ is amount of TdR in DNA.

Table xvii

TYPE OF TREATMENT - Normal

Time (hrs.)	Synchronised with Colcemid		Synchronised without Colcemid*	
	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.
2	1.43	0.07	3.34	0.42
3	1.85	0.03	3.52	0.10
4	1.76	0.16	4.70	0.06
5	1.57	0.09	7.07	0.66
6	2.45	0.09	10.32	0.28
7	3.68	0.76	14.79	0.28
8	4.95	0.35	17.28	1.32
9	11.47	0.49	23.49	2.86
10	10.98	0.67	33.82	1.74
11	20.99	1.80	39.59	1.61
12	28.41	2.17	50.56	2.06
13	31.33	2.48	45.86	2.49
14	35.90	3.43	52.52	0.88
15	36.85	3.29	55.43	0.78
16	34.50	1.04	57.77	1.48
17	34.30	1.54	58.87	4.47
18	40.52	1.46	55.00	2.04
19	41.64	0.94	57.81	2.10
20	44.87	2.09	54.67	2.20
21	59.09	4.96	59.92	1.51
22	59.24	6.67	61.92	1.31
23	55.61	3.89	63.89	1.53
24	59.64	7.31	65.72	3.29
25	64.80	9.60	71.48	1.07
26	70.74	4.67	78.43	1.70
27	77.15	1.16	78.41	1.99
28	81.98	3.63	82.23	2.58

*Obtained from G. Miller (1971)

Table xviii

TYPE OF TREATMENT - Unifilar 5-BUdR

	Synchronised with Colcemid		Synchronised without Colcemid	
Time (hrs.)	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.
2	4.50	1.24	1.84	0.11
3	5.50	0.87	4.23	0.03
4	3.73	0.45	3.87	0.64
5	4.30	0.67	4.83	0.47
6	5.57	1.65	4.64	0.52
7	7.27	0.83	9.57	0.58
8	9.94	1.02	11.36	1.40
9	19.17	5.30	12.15	0.65
10	31.53	4.37	11.81	1.20
11	22.39	2.44	23.84	1.50
12	29.59	1.50	33.62	1.66
13	41.69	9.47	38.85	3.48
14	53.25	6.32	41.56	1.82
15	62.43	4.74	49.70	3.10
16	71.19	2.80	51.75	3.27
17	74.42	1.96	61.02	2.12
18	57.71	0.53	62.26	2.32
19	89.40	1.77	95.78	2.31
20	93.10	3.48	90.56	1.95

Table xix

TYPE OF TREATMENT - Bifilar 5-BUdR

	Synchronised with Colcemid		Synchronised without Colcemid	
Time (hrs.)	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.
2	10.29	2.31	4.97	1.18
3	6.31	0.75	5.68	0.65
4	6.71	0.68	5.29	1.02
5	3.77	0.37	4.43	1.00
6	5.01	0.46	5.50	0.44
7	12.49	0.13	13.65	1.42
8	12.52	1.87	7.44	0.35
9	8.81	1.62	12.36	2.52
10	14.81	4.25	16.44	3.26
11	22.60	3.96	19.83	1.18
12	29.48	3.40	30.20	1.42
13	36.50	1.84	41.19	2.98
14	30.71	1.69	43.84	3.54
15	39.90	1.25	48.22	6.52
16	41.27	8.67	50.52	0.58
17	54.60	2.99	60.03	2.90
18	54.08	2.33	62.37	1.50
19	60.58	0.78	72.03	0.58
20	88.70	6.00	56.29	0.44

Table xx

TYPE OF TREATMENT - Unifilar TdR

	Synchronised with Colcemid		Synchronised without Colcemid	
Time (hrs.)	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.
2	3.06	0.75	2.94	0.45
3	3.79	0.10	3.37	0.23
4	2.46	0.15	4.82	0.12
5	3.86	0.56	5.65	0.55
6	3.35	0.42	7.45	0.12
7	3.97	0.30	10.16	0.93
8	7.57	0.89	18.15	2.11
9	9.74	1.88	25.42	0.67
10	14.70	1.63	28.98	3.56
11	21.85	3.10	40.99	0.89
12	28.70	5.43	44.90	0.51
13	39.73	1.06	60.81	4.15
14	43.10	3.60	57.65	5.49
15	52.71	6.40	64.87	2.15
16	64.20	0.94	73.68	0.67
17	68.80	3.41	82.66	4.14
18	69.70	1.70	83.73	2.64
19	79.70	4.10	91.65	3.64
20	90.90	4.50	95.39	2.31

Table xxi

TYPE OF TREATMENT - Bifilar TdR

	Synchronised with Colcemid		Synchronised without Colcemid	
Time (hrs.)	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.	Mean Relative Count/Min.	Standard Error of Mean Relative Count/Min.
2	1.60	0.03	3.94	0.83
3	2.89	0.74	9.17	1.33
4	4.17	1.33	4.81	0.61
5	3.75	0.06	6.01	2.41
6	4.05	0.87	5.69	0.57
7	6.53	1.00	12.77	0.85
8	12.76	1.86	14.33	4.55
9	19.29	1.96	31.69	1.99
10	21.76	1.74	43.45	1.70
11	30.78	0.82	46.59	8.10
12	34.22	8.52	49.60	0.21
13	42.66	1.23	69.02	3.11
14	52.55	3.42	46.52	1.76
15	56.14	3.06	60.89	3.07
16	62.01	5.93	70.57	3.11
17	68.65	0.50	74.14	4.36
18	85.78	8.48	77.51	4.52
19	89.55	5.68	91.10	5.55
20	76.98	5.22	79.38	6.31